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Medicinal plants and their secondary metabolites – State of the art and trends in breeding, analytics and use in feed supplementation – with special focus on German chamomile

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Summary

Plants in ecology interact with their biotic and abiotic environment. Means of interactions, and thereby crucial for the survival of the species, are secondary plant metabolites. Exploration of the plants' metabolism in action and reaction to their environment, the formation and release of secondary plant metabolites in specific (stress) situations and their functions as attractants, defence or protection agents gives us the chance to exploit the knowledge for human purposes. The purpose of medicinal use of these metabolites turns the bearing plants into medicinal plants. Health promoting, disease preventing and other favourable effects make these plants and their extracts valuable as dietary and feed additives. This review covers some current aspects of conventional breeding, particularly of German chamomile, of phytochemical analytics and of use of medicinal plants in feed supplementation. The main three groups of functional plant metabolites terpenes, polyphenols and alkaloids are treated. Outlook in research, in challenging analytical questions and in effective product development is provided, outlining current trends in production of secondary plant metabolites and demands on the market.

Keywords: *Matricaria recutita*, terpene, alkaloid, polyphenol, controlled crossing, male sterility

Introduction

Usually, plant derived compounds are classified into primary and secondary metabolites, according to their function in living plants. While primary metabolites are crucial for general living of the plant and are produced equally by all representatives of plant kingdom, so-called secondary metabolites or biologically active substances are often plant family-specific, ecologically valuable metabolites, making survival and reproduction under specific environmental conditions and interactions successful (Abbas et al., 2017). These metabolites include e.g. attractants for pollinators, repellents and anti-feedants against herbivores, anti-microbial substances against pathogen attacks or protective substances in case of abiotic stress. While these compounds are of immense ecological advantage for the living plant itself, there is also a huge potential to use biologically active plant metabolites by humans (Gutzeit and Ludwig-Müller, 2014; Bustos-Segura and Foley, 2018).

Medicinal plants are the most common source of active substances, including mainly alkaloids, terpenes, polyphenols and others (Gutzeit and Ludwig-Müller, 2014; Van Wyk et al., 2015). Domestication, cultivation and breeding efforts of plant species, suitable to deliver these compounds as intact plant material or as extract, turn these plants into crops (Franke, 2009). Still, medicinal plants belong to the underutilised crops, with a high need to investigate genetic and metabolic profiles. Ideal cultivation and harvesting practise and effects on human and animal health or their potential for further purposes, like for use in cosmetics or in plant protection, have to be explored (Pank and Blüthner, 2009; Faehnrich et al., 2019c).

Breeding approaches aim to enhance the content of specific compounds or to reach a certain composition of compounds in stable breeding lines, as well as to establish certain flowering behaviour or seed set to meet agronomical needs (Faehnrich et al., 2019b). To ensure qualitative and quantitative occurrence of valuable ingredients – mostly a whole bouquet of substances – phytochemical, chromatographic and photometric analytics of liquids and volatiles are necessary, as well as microscopic analyses of solid plant material (Petruczynik, 2012; Csepregi et al., 2016). Finally, the biologically active substances of medicinal plants mostly stay active after application or intake by humans or animals. The most interesting aspect of investigations therefore is, if, how and to which extent these ingredients work. Where are the potentials and where are the risks of the use of medicinal plant material (Brenes et al., 2016; Faehnrich et al., 2016a; Chen et al., 2018)?

Products of secondary plant compounds gain presence on the market. This development may occur on the one hand due to an increasing interest in natural products and on the other hand due to legal bans on the use of synthetic chemicals, e.g. of neonicotinoids in plant protection or of anti-biotics as growth promoters in animal production (Pino et al., 2013; Van Wyk et al., 2015). Therefore, investigations on all aspects of biologically active metabolites of medicinal plants are a growing and highly appreciated research field.

This review will give an overview of the relevance of secondary plant metabolites and will cover recent aspects of conventional breeding, analytics and use of medicinal plants, concentrating on feed additives. Focus lays on specific and prevailing challenges of research, like establishing male sterility for breeding purposes or analysing polyphenolic flavonoids. However, future prospects will touch new and important developments in production and use.

Biologically active (secondary) metabolites in plants

In nature, secondary plant metabolites have diverse ecological functions. They are involved in all interactions of plants with their environment and crucial for their defence and their reproduction mechanisms (Abbas et al., 2017). Fig. 1 gives an overview of some important tasks of secondary plant metabolites. Plant physiology and phytochemistry help to elucidate their production, their deposition, and their metabolism (Zeng et al., 2013). The knowledge of their tasks and of biosynthetic pathways will be the base for breeding, analytics and use of medicinal plants (Pino et al., 2013; Gutzeit and Ludwig-Müller, 2014).

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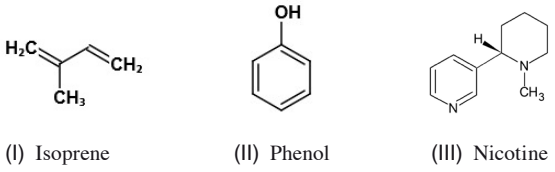

(I) Isoprene (II) Phenol (III) Nicotine

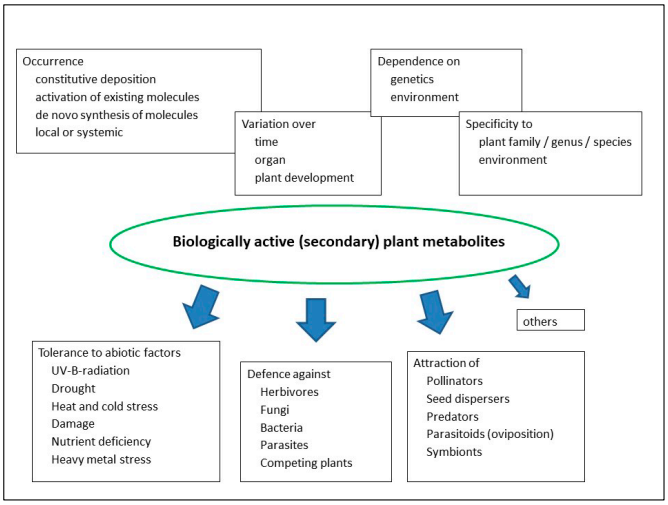

Fig. 1: Overview of factors of occurrence and of main roles of secondary plant metabolites in natural ecology. Different general ways of biosynthesis/activation of secondary metabolites, depending on their designated tasks, are possible, while plant specific content and composition of secondary compounds may vary due to diverse influences.

The three main groups of secondary plant metabolites can be defined as: terpenoids, (poly)phenols and alkaloids (Pino et al., 2013).

Terpenes and the isomeric terpenoids are compounds deriving from isoprene units (I). They are often volatile and play crucial roles in ecological communication. They can be released by the plant or elute after mechanical or herbivore induced damage and are included in plants' defence strategies (Bustos-Segura and Foley, 2018). For the efficacy and the informative value of released essential oils of plants, the composition of terpenes is crucial, influenced by the occurrence of specific intrinsic enzymes or by enzymes in the herbivorous saliva. Often the released composition of terpenes attracts predators of the attacking herbivore or act as signals for other plants to induce defence mechanisms (Bede et al., 2006).

Polyphenols are astringent, mostly soluble and anti-oxidative polymers of phenol structural units (II). They are involved in the protection against UV-B-radiation, are important pigments for fruit and flower colours, are, therefore, crucial for effective pollination and seed dissemination (e.g. flavonoids, anthocyanins), defend the plants against herbivores (e.g. tannins), give strength to plant tissues against mechanical damage, and keep off micro-organisms (e.g. lignins) (Faehnrich et al., 2016a).

Alkaloids, complex N-containing organic compounds, are defence-related metabolites, which can accumulate in case of pathogen or herbivorous attacks, like the accumulation of nicotine (III) in *Nicotiana attenuata* (Onkokesung et al., 2010). During plant development, physiological and chemical adaptation causes a change in alkaloid structure and a change in deposition sites, like the rearrangement from the benzylisoquinoline alkaloid protopine to sanguinarine in *Macleaya cordata* and the accumulation of the latter in leaves during vegetative growth and in fruit shells in times of seed formation (Zeng et al., 2013).

Besides these three main groups of secondary plant metabolites, there are many other molecules serving as effective secondary plant metabolites. Examples are some amino acids, e.g. β-aminobutyric acid, an elicitor in plant resistance reactions (Cohen et al., 2016) or canavanine, a poison against herbivores (Krasuska et al., 2016). Also unchained hydrocarbons, e.g. the phytoalexin falcarinol (Charles et al., 2008), glycosides, e.g. cardenolides in foxglove plants, which increase in case of wounding and/or abiotic stress (Perez-Bermudez et al., 2010), and others (Macias et al., 1998) might belong to the functional plant compounds. Though extremely interesting, they will not be treated further in this work, as we have to confine the extent.

Breeding of medicinal plants

History and breeding aims

Since ancient times, people collected plants, plant material with functional compounds from the wild, and used them for medicinal purposes (Jamshidi-Kia et al., 2018). In tropical areas, where the biodiversity, the number of different plant species and the biotic stress is higher than in moderate climates, it can be assumed, that, in response, also the content and the variability of secondary plant compounds are higher (Andresen et al., 2018). In various cultures, traditional healers maintained and transferred the knowledge of therapeutic properties and modes of application from generation to generation. The eldest written evidence of use of medicinal plants is known from China, Egypt and India, dating back to Sumerian documentations on clay tablets nearly 5000 years ago (Khan, 2014).

Wild collection of medicinal plants is still done in some countries and for some plants, but the trend, driven by an increasing demand on quantity and quality quickly led to domestication and efficient cultivation. While the first attempts of cultivation only comprised collection of living plant material or seeds to grow them on a permanent location, breeding started in the moment of selection of plants with certain features (Franke, 2009; Das, 2015).

Recent breeding aims of medicinal plants include, but are not limited to:

(i) High content of desirable compounds and/or absence of harmful substances (e.g. *Artemisia annua*/ *A. umbelliformis*), stability of quality
(ii) High and homogenous yield of the harvested plant parts or products (e.g. *Thymus vulgaris*), low input/high output ratio
(iii) Morphological improvements to optimize gain of desired product like size or number of flowers (e.g. *Matricaria recutita*), proportion of ray to disk flowers (e.g. *Calendula officinalis*), root structure (*Valeriana officinalis*), resistance of oil glands (e.g. *Mentha* × *piperita, Salvia officinalis*)
(iv) Resistance to biotic (pests and diseases) and abiotic stress (drought, salt stress, temperature, etc.) (e.g. *Hypericum perforatum*)
(v) Suitability for mechanical harvesting and other agronomical requirements like fast youth growth, equal flowering horizon, synchronous maturity (e.g. *Matricaria recutita*), and resistance to disintegration of flower heads or double achenes (e.g. *Coriandrum sativum*), resistance of grain shedding, upright growth (e.g. *Plantago lanceolata*) (Carlen, 2012; Faehnrich et al., 2014; De, 2017; Faehnrich et al., 2019b; Penzkofer, 2019).

Conventional breeding

Botanical basics

Basic botanical investigations to explore physiology of production of secondary metabolites and their accumulation in plant organs and cell compartments are crucial prerequisites for successful breeding on medicinal plants. Fig. 2 gives an example for the location, particularly of essential oils, in multi-cellular glandular trichomes. Fig. 3-6 show detailed generative structures, like hermaphroditic flower for-

mation in Asteraceae, pollen tube and embryo formation after fertilization. Furthermore, compatibility of crossing partners, occurrence of sterility and ability for vegetative and generative propagation are in the focus of current breeding research (FAEHNRICH et al., 2016b).

Controlled crossing

In generative breeding, controlled crossings are indispensable, be it for simple combination of properties and/or increase of variability or for creating hybrid or synthetic cultivars (PANK and BLÜTHNER,

Fig. 2: Multicellular glandular trichome (in the circle) on the inferior ovary of *Matricaria recutita*, consisting of two base cells, two peduncle cells and a secretory head, as one of the main storage organs containing the essential oil. At the bottom of the ovary a ring of sclerenchyma stone cells as a predetermined breaking point from the receptacle.

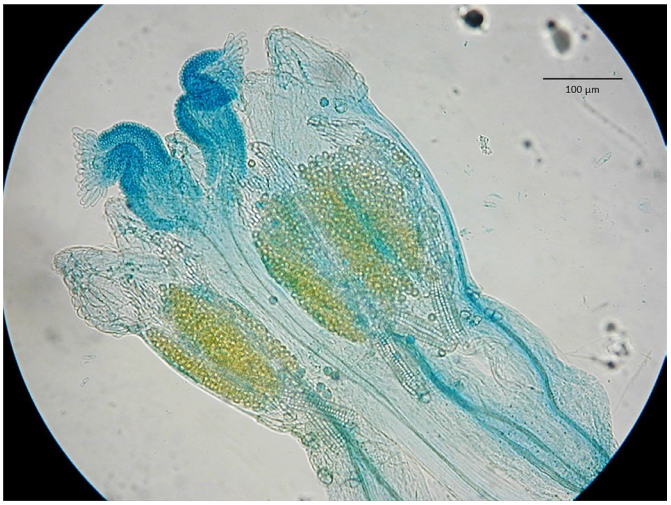

Fig. 3: Corolla of a disc flower in *Matricaria recutita*, composed of five merged petals. Hermaphroditic floret, containing five stamens with a huge number of pollen and the pistil with a two-branched stigma and papillae.

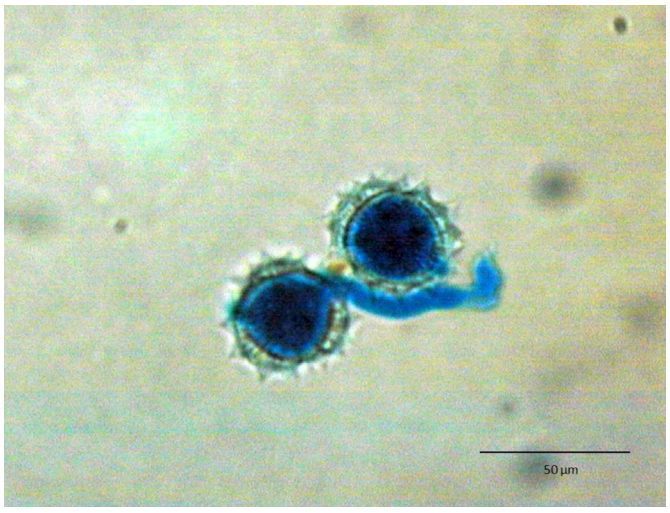

Fig. 4: Spiny exine, intine and cytoplasm of pollen grains of *Matricaria recutita* with a pollen tube growing out of one of the pores, stained with lacto-phenol-aniline blue solution under light microscope.

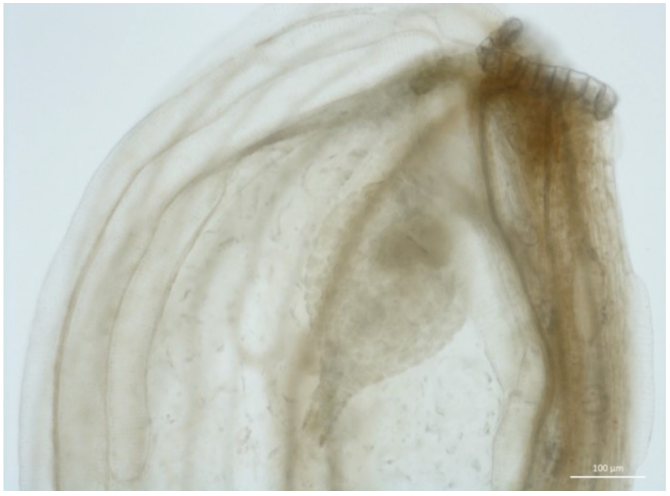

Fig. 5: Embryonic development in the heart stage surrounded by the multicellular endosperm in the embryo sac of the anatropous ovule in *Matricaria recutita*.

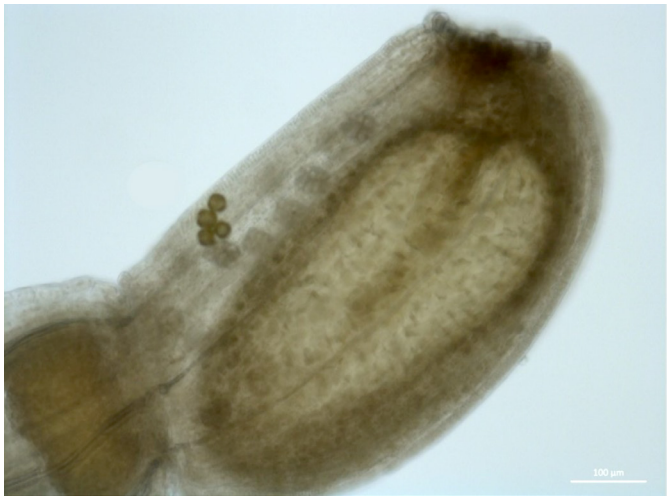

Fig. 6: Embryonic development in the cotyledonous stage with already bent over cotyledons after consumption of most of the endosperm, near to ripeness of the seed and the achene.

2009). In some cases open pollination within a population will be constructive (e.g. to evaluate the response to previous selection in the daughter generation (F_1) compared to the initial population), in some cases individual genotypes will be crossed for subsequent selection in the F_1 (FAEHNRICH et al., 2019b). Sometimes, even controlled self-pollination is desired, e.g. to test cytoplasmic and/or genetic male sterility (OTTO et al., 2016). Using hermaphroditic plant material, one of the main challenges is to succeed in controlled crosses without self-pollination (FAEHNRICH et al., 2016b). Approaches to reach this aim are through (BECKER, 1993):

(i) Self-incompatible (SI) mother lines
(ii) Maternal genetic or cytoplasmic male sterility (CMS)
(iii) Systems of emasculation (manually or chemically) in mother lines

To prove success or failure of establishment of self-incompatibility and to select potential SI candidates in mainly cross-pollinating plants, in conventional breeding systems two methods are common (FAEHNRICH et al., 2015):

(i) Determination of (viable) seed set on mother plants after isolation
(ii) (Fluorescence) microscopic evaluation of growth of pollen tubes in stigma and style and embryonic development in the ovary, resp. (Fig. 4-6) after isolation

To prove pollen viability/sterility after means of emasculation or to select suitable candidates of spontaneous or induced, genetic or cytoplasmic male sterility, an aceto-carmine staining and light microscopic evaluation is appropriate (YANKOVA-TSVETKOVA et al., 2013; YU et al., 2017).

After reaching/finding self-incompatible or (cytoplasmic) male sterile plants, their conservation by vegetative maintenance or by generative systems will be necessary (FAEHNRICH et al., 2016b).

Plant genome comprises three parts: The nuclear, chromosomal genome, mainly responsible for protein and RNA biosynthesis, and the far smaller, in Embryophyta circular, genomes of the plastids (plastome) and the mitochondria (chondriome) which also take over special functions (WEILER and NOVER, 2008; KADEREIT et al., 2014). While the plastome, for example, encodes for proteins involved in photosynthesis, the disposition of mitochondrial genes in combination with nuclear genes is decisive for the development of maternally inherited CMS. In this case, a male fertile maintainer is necessary to pollinate a CMS mother line and create again CMS offspring. In case of medicinal products, deriving from seeds (e.g. *Linum usitatissimum, Carthamus tinctorius*) an additional restorer line can recreate male fertility and intact seed production in the consecutive generation. Sometimes, the vegetative parts of the plant (sepals, petals, leaves, roots, etc.) are the harvest products, therefore, only maintainer lines to maintain once established CMS lines are necessary (PANK and BLÜTHNER, 2009).

To maintain self-incompatibility, by vegetative (e.g. from cuttings) or by generative (from seeds) propagation, specific investigations for the respective species/variety have to be performed in order to evaluate environmental and genetic influence and to assess heritability (WATANABE et al., 2012; FAEHNRICH, 2016b).

Genomic alteration

The basic number of genomes in somatic cells (ploidy) is crucial for the phenotype, as increasing ploidy level mostly entails larger cell sizes, often also larger, but fewer flowers/flower heads, larger seeds and sometimes delayed or extended florescence (OTTO et al., 2015). In various medicinal plants, these features increase yield, e.g. of essential oil or of the herbal drug, or widen the harvesting period. Therefore, allo- and autopolyploid lines are bred (Fig. 7; FAEHNRICH et al., 2019b).

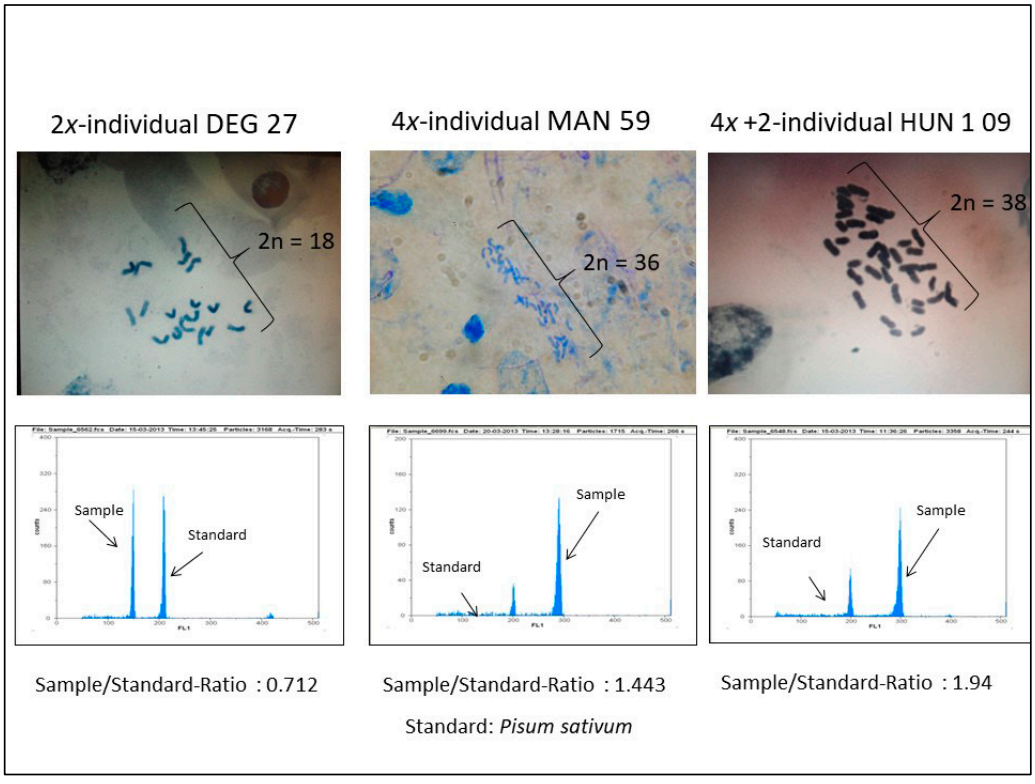

Fig. 7: Light microscopic chromosome counts and histograms of flow cytometry of three individuals of *Matricaria recutita*, showing sample: standard fluorescence ratios of a eu-diploid (DEG 27, 18 Chromosomes), a eu-tetraploid (MAN 59, 36 chromosomes) and a hyper-tetraploid (HUN 1 09, 38 chromosomes) individual. The basic chromosome number is x = 9 (Source: FAEHNRICH et al., 2019b).

To create auto-polyploid forms, treatment with mutagenic chemicals like colchicine, the toxin of the autumn crocus (*Colchicum autumnale*), has been conducted in various cases in medicinal plants. Examples for colchicine-induced polyploids are the auto-tetraploid cultivar 'Manzana' of German chamomile, and the polyploid cultivar 'Multimentha' of peppermint (LAWRENCE, 2009; PANK and BLÜTHNER, 2009).

Interploid crossings are a common way to generate anorthoploids, which might, due to impaired chromosome numbers, develop seed sterility or complete lack of seeds. This, in turn, can be linked to agronomically desired features like long flowering time or the absence of volunteers in the following crop (OTTO et al., 2015).

Besides the mentioned common ways to alter genomes in medicinal plants, gene, chromosome, and genome mutations can be induced by ionizing radiation or by mutagenic agents, as well as by somaclonal variation occurring after *in-vitro* tissue culture. These mutations can be used to increase variability, to select sought for characteristics or to develop pathogen resistant genotypes (PANK and BLÜTHNER, 2009; FRANZ and NOVAK, 2016).

Physiological intervention by plant hormones

Plant hormones (avoiding the expression 'phytohormones', as these also can be understood as secondary plant metabolites, working in animals' or human bodies like intrinsic hormones) are signal molecules, produced in plants and influencing all kinds of physiological growth and development processes in plants. Important plant hormones include abscisic acid, auxins, cytokinins, ethylene, gibberellic acid, jasmonates, salicylic acid and others (ASAMI and NAKAGAWA, 2018). Some of them are commonly used in plant breeding and propagation of crops. Nevertheless, in some cases, due to the focus on secondary metabolites as valuable gain, treatment with plant hormones particularly shall increase these values (GUPTA et al., 2018; ZABARJADI et al., 2018) or on the other hand, secondary metabolites are precursors for hormone biosynthesis, like carotenoids for abscisic acid (AIHUA et al., 2018). The exogenous application of plant hormones for production of valuable metabolites can be found in biotechnological and genetic engineering approaches (WASTERNACK and STRNAD, 2019). Moreover, genetic engineering of plants might affect genes involved in hormone release (e.g. jasmonates) which, again, activate secondary metabolism (ABBAS et al., 2017). This approach cross-links interests in secondary metabolites as medicinal values and as effective agents in terms of plant protection (MACIAS et al., 1998; LIU et al., 2017). Tab. 1 shows examples of breeding research on the interaction of plant hormones and secondary plant hormones.

Analytics of medicinal plants

Since the medicinal effect of plant material mostly derives from the secondary metabolites, phytochemical analytics concentrate on assessment and evaluation of terpenoids, alkaloids and polyphenols.

Terpenes

Terpenes are commonly classified into mono-, sesqui-, di-, tri- and tetra-terpenes. Two isoprene-units (I, i.e. 10 C-atoms) form one terpene unit. Units are combined in an aliphatic (e.g. myrcene) or in a cyclic (e.g. bisabolene) way. Terpenoids are even more complex, mostly multi-cyclic compounds, which derive from terpenes. They, additionally, contain diverse, mostly O-bearing, functional groups (i.e. alcohols, aldehydes, ketones or esters).

Essential oils mainly consist of semi- (e.g. 2-methyl-3-buten-2-ol), mono- (e.g. linalool, thymol), sesqui- (e.g. bisabolol, chamazulene), and a few diterpenoids (e.g. sclareol) (FAEHNRICH et al., 2014; SICARI and POIANA, 2017). Besides terpenoids, also volatile phenylpropanoids (e.g. eugenol, apiol), fatty acid derivatives (e.g. hexenal) and amino acid derivatives (e.g. 2-phenyl-acetaldehyde) occur in essential oils (TIRILLINI et al., 2004; ROUT et al., 2007).

Gas chromatographic (GC) analysis is the method of choice to analyse occurrence of volatile compounds. Head space – solid phase micro extraction (HS-SPME) is a suitable preceding extraction method for volatiles, binding even very low amounts of the analytes on an adhesive fibre coating (FAEHNRICH et al., 2017). To determine and quantify a specific terpenoid compounds, GC is combined with mass spectrometry (MS) and with flame ionisation detection (FID) (FAEHNRICH et al., 2014; ZRIBI et al., 2019). Ion-spectra are matched with external (ADAMS, 2007) and online (NIST, Wiley) databases. Fig. 8 shows eucalyptol (syn. 1, 8-cineole) as a natural, hetero- and bicyclic monoterpenoid, abundant in many medicinal plants like *Eucalyptus* spp., *Rosmarinus officinalis* and *Salvia officinalis*.

In aromatic and medicinal plants, a complex profile of terpenes is expected. Thirty and more free, oxygenated and glycosidically-bound terpenes contribute to a volatile and aromatic bouquet (TIRILLINI et al., 2004). The profile can vary substantially in different environments, developing and ripening stages, besides differences due to plant genotype. Under different climatic conditions, transcription factors might trigger different enzymatic processes for terpene accumulation (ZRIBI et al., 2017).

However, terpenoids are not confined to the volatile, low molecular weight compounds, but can also consist of six isoprene-units (I) (C30, tri-terpenoids, e.g. squalene, phytosterols) and eight isoprene units (I) (C40, tetra-terpenoids, carotenoids). These terpenoids, being hydrophobic and lipophilic, are not volatile under usual definitions

Tab. 1: Plant hormones in breeding of medicinal plants.

Plant hormone	Use in breeding of medicinal plants	Plant species	Source
Abscisic acid	To explore influence on micro-RNAs involved in production of secondary metabolites	*Persicaria minor*	NAZARUDDIN et al., 2017
Auxin	To overcome endosperm incompatibilities in interploid crossings	*Matricaria recutita*	FAEHNRICH et al., 2019b
Cytokinin	To induce shoot production in endosperm tissues	*Passiflora edulis*	ANTONIAZZI et al., 2018
Ethylene	To induce chemical emasculation	*Matricaria recutita*	FAEHNRICH et al., pers. comm.
Ethylene	To increase flavonoid and isoflavonoid production	*Glycine max*	GUPTA et al., 2018
Gibberellic acid	As a gametocide for chemical emasculation of mother lines	*Matricaria recutita*	FAEHNRICH and FRANZ, 2012
Gibberellic acid	To increase content of secondary metabolites (artemisinin)	*Artemisia annua*	ZEBARJADI et al., 2018
Jasmonate	To increase parthenolide production	*Tanacetum parthenium*	MAJDI et al., 2015
Salicylic acid	To increase production of secondary metabolites (anthraquinones)	*Aloe vera*	LEE et al., 2013

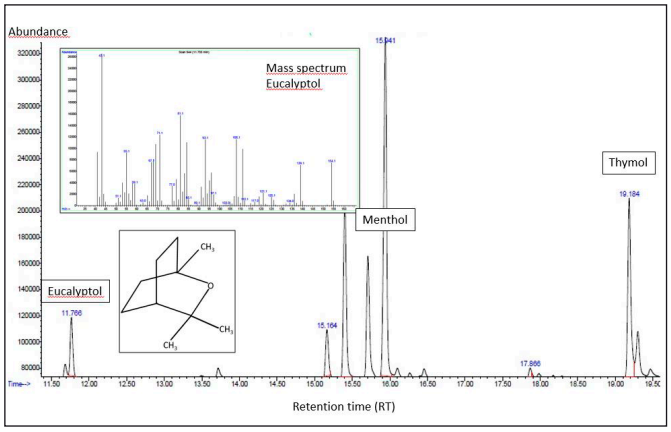

Fig. 8: Occurrence of eucalyptol in a gas chromatographic chromatogram (RT 11.766) of a blend of volatile compounds of a fresh essential oil bearing feed supplement, referring mass spectrum and chemical structure of eucalyptol (Source: FAEHNRICH et al., 2017, modified).

(eluting at room temperature) and are therefore analysed by liquid chromatography methods (high performance liquid chromatography (HPLC), liquid chromatography with tandem mass spectroscopy (LC-MS/MS)) or semi-quantitatively by thin layer chromatography (TLC) (PRIYA et al., 2016; POIRIER et al., 2018).

Polyphenols

Polyphenols are defined as a group of compounds, which have more than one phenolic hydroxyl group on one or more benzene rings (II). These compounds derive from the shikimate/phenylalanine biosynthetic pathway in plants, separated by the activity of specific enzymes and include stilbenes, lignins, coumarins and flavonoids. Flavonoids, the largest group, can be subdivided into flavones (e.g. apigenin), flavonols (e.g. kaempferol), and isoflavonoids (e.g. genistein). Flavonols also include anthocyanins and condensed tannins or proanthocyanidines (GUTZEIT and LUDWIG-MÜLLER, 2014). Side branching and complexation with metals or sugars change their appearance and functionality (FAEHNRICH et al., 2016a).

Phenylpropanoids are also mediate products of polyphenol synthesis, appearing as acids (e.g. cumaric acid, cinnamic acid), alcohols (e.g. *p*-coumaryl alcohol) or aldehydes (e.g. cinnamaldehyde). They can aggregate to complex phenylpropanoids like rosmarinic acid. Hydrolysable tannins (gallotannins) are esters of phenolic acids and sugars (GUTZEIT and LUDWIG-MÜLLER, 2014; VAN WYK et al., 2015). As (poly)phenols vary widely in their structural composition, also their physicochemical properties vary and furthermore, the methods of detection and quantification. Phenolics are in general relatively hydrophilic, and are extracted using water or polar organic solvents (TSAO, 2010). The focus in here is laid on flavonoids, with a basic chemical structure of a C15-frame consisting of two aromatic rings connected by a third O-including ring (FAEHNRICH et al., 2016a). Flavonoids in plant material can occur in the free form or glycosylated with a sugar moiety. Glycosylated flavonoids are less reactive, more polar and more water soluble than free aglycones (CORRADINI et al., 2011; Fig. 9).

To quantify single flavonoids, the common method is reversed-phase high performance liquid chromatography (HPLC), coupled with a UV-detection by a diode array detector (DAD) (TSAO, 2010; FAEHNRICH et al., 2014; Fig. 9).

As flavonoids, like all polyphenols, are prone to easy alteration due to oxidation, pH-change, complexation and co-pigmentation, etc., sometimes the best way of examination might be to assess the whole flavonoid content at once without separation of single compounds

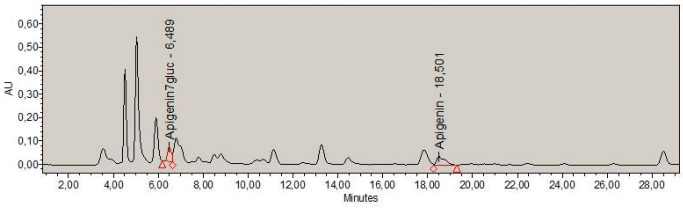

Fig. 9: HPLC-Chromatogram of a methanolic extract of dried flower heads of *Matricaria recutita*, highlighting the apigenin-7-glycoside and the (free) apigenin peaks at the retention times of 6.49 and 18.50 minutes, respectively. AU = Absorption units

(FAEHNRICH et al., 2016a). Determination of total flavonoids generally is done by a colorimetric method, where extracts are mixed with a complexing aluminium chloride solution. A photometric microplate reader records the absorbance at a certain wavelength. A calibration curve is generated with a pure reference polyphenol, like rutin or quercetin. Results are expressed as equivalents of the reference flavonoid (CHANDRA et al., 2014; SALEHI et al., 2018)

Alkaloids

Alkaloids are nitrogen-containing and often heterocyclic compounds, which are mainly found in plants. Their basicity and salt-building reactions with acids give them their name (DEWICK, 2009). In their pure form, most alkaloids are crystalline solid, colourless and non-volatile. Nevertheless, some are coloured, as sanguinarine (orange) or berberine (yellow) and some oxygen-free alkaloids appear as oily liquids (nicotine (III), coniin). Most of the alkaloids have a bitter taste (KUKULA-KOCH and WIDELSKI, 2017).

Some plant families like nightshades (Solanaceae) and the poppy family (Papaveraceae), particularly, are rich in different alkaloids. In other cases, certain plant species present the occurrence of a typical alkaloid (e.g. *Coffea sp.* / caffeine, *Conium maculatum* / coniine) (HARBORNE, 1984; GUTZEIt and LUDWIG-MÜLLER, 2014). Often, the relationship of species can be proven by the occurrence of the same or similar alkaloids (chemotaxonomy) (HOLM and HERBST, 2001). Alkaloids can be classified according to the N-position and -number in their chemical structure (indole alkaloids (e.g. strychnine), tropane alkaloids (e.g. scopolamine), isoquinoline alkaloids (e.g. sanguinarine), phenylethylamine alkaloids (e.g. colchicine), pyrrolizidine alkaloids (e.g. senecionine), xanthine alkaloids (e.g. caffeine), pyridine alkaloids (e.g. nicotine, III) (DEWICK, 2009; PETRUCZYNIK, 2012). Because alkaloids have generally a strong physiological effect on mammalians, plants containing alkaloids often are used as medicinal plants (e.g. *Papaver somniferum*, *Cinchona pubescens*, etc.) (HOLM and HERBST, 2001; NORDEGREN, 2002).

Currently applied analytical methods for alkaloids:

(i) Thin Layer Chromatography (TLC) is appropriate rather for qualitative than for quantitative determination of alkaloids. The separation of compounds according to their polar/nonpolar nature, e.g. by using a polar solid phase (e.g. TLC plate of silica gel) and a solvent of medium polarity, provides a kind of fingerprint of a certain plant extract (SPANGENBERG, 2014). In comparison with pure standard substances in different concentrations, a semi-quantitative estimation of the occurring compounds can be given. Alkaloid bands in the chromatogram can be detected at different wavelengths (254 nm, 365 nm) and/or after spraying with suitable reagents (WAGNER and BLADT, 1996). Combined with densitometry, the results can be converted into semi-quantitative results (PETRUCZYNIK, 2012).

(ii) High performance liquid chromatography (HPLC) combined with detection systems of UV-absorption (e.g. photodiode array detector, DAD), of mass spectrometry (MS), of infrared absorp-

tion (IR), of nuclear magnetic resonance (NMR) spectroscopy and others give accurate quantitative results of single occurring compounds (NAM et al., 2016; PICRON et al., 2018). Various adaptations and refinements of the HPLC procedure (normal (NP) or reverse stationary phase (RP), ultra-performance liquid chromatography (UPLC), ion-exchange chromatography (IEC), micellar liquid chromatography (MLC), hydrophilic interaction chromatography (HILIC), and others) are possible and can be applied for appropriate analysis of certain alkaloid compounds (PETRUCZYNIK, 2012).

Furthermore, there are still simple colorimetric and titrimetric reactions with diverse reagents in use, which can give an estimation of alkaloid-content of plant extracts (HOLM and HERBST, 2001). Some reagents (e.g. Dragendorff reagent) are nowadays rather applied as spray for TLC visualization (HARBORNE, 1984).

Analytical challenges

Bouquet of compounds

In medicinal plants, mostly not a single compound is of value, but the whole bouquet of compounds together synergistically forms the scent, the taste, the medicinal effect or even the colour. Lead compounds as quality criteria in drugs (pharmaceutical, dried plant material) are sometimes the active substance itself, but more often, as the pharmaceutical effect bases on the whole profile of compounds, the lead is a compound that easily can be detected analytically and appears in a stable ratio or reaction with the others (NAM et al., 2016). Furthermore, by a proven lead substance it can be concluded that a certain plant is included in a drug mixture (SCHILCHER, 2009).

Even the definition of the active substance might change according to new findings. For example, for a long time, hypericin was said to be the most effective substance in *Hypericum perforatum* (St. John's wort). Since the 1990s, the substance hyperforin or the also included flavonoids counted as the active agents. Since 2008, extracts with a minimum content of hypericin and flavonoids, besides a maximum content of hyperforin, were defined as the ones with the best effect (SCHILCHER, 2009).

Moreover, co-elution of compounds, fluctuating composition of compounds and very low amounts may cause further problems in plant analytics. Analytics of volatiles by solid phase micro-extraction may furthermore discriminate compounds due to their different affinity to the fibre coatings (FAEHNRICH et al., 2017). Clear peak separation and symmetry is therefore a challenge in analytics of plants' secondary compounds that is met with an appropriate choice of methods and detection techniques used for specific compounds (PETRUCZYNIK, 2012).

Effects instead of occurrence

For medicinal plants, the focus of interest will sometimes rather be on the effect instead of pure determination of compounds. Therefore, appropriate methods will explore effects and efficacy; mostly, but not always endorsed by data on analytical composition of the source material (MONTANARI et al., 2016). Effect-related tests could be:

(i) *In-vitro-tests*:
Colorimetric assays to determine the antioxidant capacity, particularly of phenolic compounds, like TEAC (trolox equivalent antioxidant capacity; CSEPREGI et al., 2016) and FRAP (ferric reducing antioxidant potential; BENZIE and STRAIN, 1996), and to test the scavenging ability for radicals by 2,2-diphenyl-1-picrylhydrazyl (DPPH) are commonly in use (SALEHI et al., 2018). Focussing overall amount of anti-oxidative active compounds in food and plant material, total phenolics can be assessed by FCR (Folin-Ciocalteu-reagent activity; CHANDRA et al., 2014). Enzymatic and microbiological *in-vitro* assays are also in use in research of plant derived secondary metabolites (LELARIO et al., 2018). Pharmacological assays using tumour cell lines as target substrate to choose suitable accessions for domestication of wild medicinal plants, without assessing the chemical composition, are described in MONTANARI et al. (2016). Usually, if microorganisms (bacteria, fungi, algae, protozoa) are the test organisms, tests are called *in vitro*-tests.

(ii) *In-vivo*-tests:
Biological tests with higher developed tests organisms may be allocated to the *in-vivo*-tests. SOBEH et al. (2018) used nematodes as *in-vivo* model organisms to determine survival rates under oxidative stress after treatment with a phenolic extract. Rats and other rodents can serve as test organisms for the efficacy of secondary plant metabolites (SOBEH et al., 2018). Tests with living animals will generally have the problem of result-influencing individuality of test objects, deriving from genetic and environmental pre-existing conditions. It may be assumed, that the higher developed and the longer living the animals are, the higher the dominance of the individuality can be. While feeding tests with rodents or fast growing poultry might rather display clear effects on the test animals, tests with cattle or horses might strongly be influenced by individual genetic and/or environmental conditions (GOODARZI and NANEKARANI, 2014; FAEHNRICH et al., 2019a).

Multivariate Evaluation

To evaluate a bouquet of compounds at once, as it is necessary to capture a whole taste or fragrance or to track an over-all effect, group-wise discrimination of chemotypes and/or multivariate evaluation is recommended (FAEHNRICH et al., 2014; FAEHNRICH et al., 2017; Fig. 10).

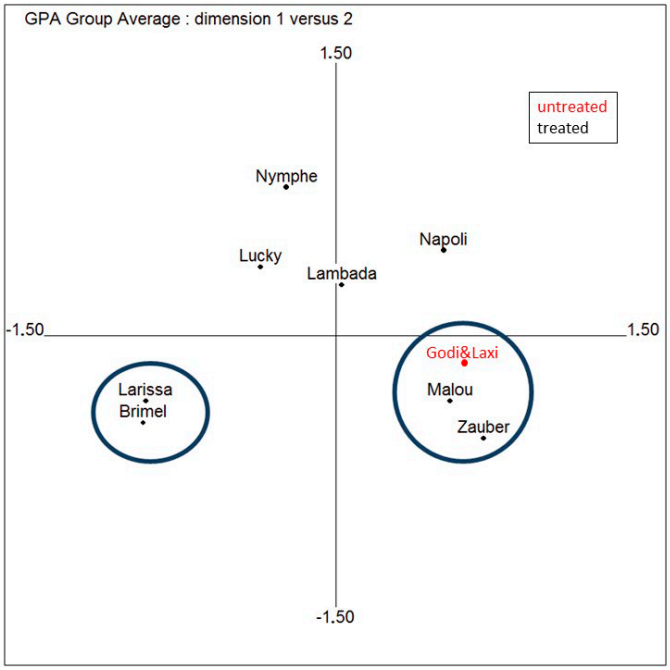

Fig. 10: Generalized procrustes analysis (GPA) of free-choice profiling data of a cheese sensory test of milk of eight cows fed with a phytogenic feed supplement in comparison with untreated control cows (*Godi* & *Laxi*). Bi-plot showed similar sensory properties of cheese of control cows to samples from treated cows (*Malou* and *Zauber*). Furthermore, similar taste occurred with cheese samples from *Larissa* and *Brimel*. A clear separation of samples from treated or untreated cows was not revealed (Source: FAEHNRICH et al., 2017, modified).

Even if the single compounds are not determined as such, phytochemical and/or genetic fingerprinting (e.g. by amplified fragment-length polymorphism, AFLP), providing a characteristic pattern, is indispensable to compare useful plant sources (Fig. 11). These sources can be batches of drugs, defined plant material from certain cultivars/accessions of medicinal plants or single plants. Often, the latter is recommended, as hermaphroditic, mostly outcrossing plants will form genetically heterogeneous populations and therefore will not be uniform in their chemical composition. This is particularly essential in breeding and selection of suitable parental plants for crossings (BEC et al., 2021).
Multivariate analysis is also the means of choice to combine complex data of plant metabolites with complex *in-vivo* reactions of target organisms in systems biology approaches (VERPOORTE et al., 2005).

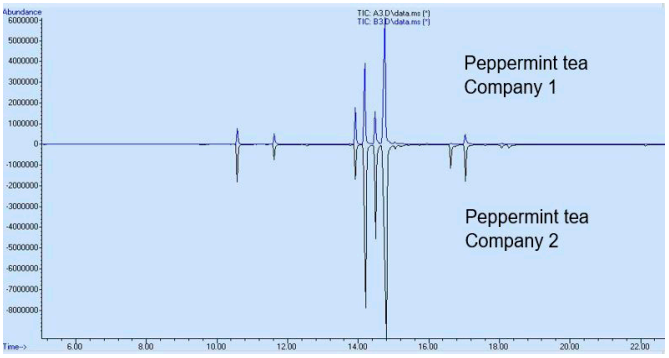

Fig. 11: Gas chromatographic comparison of two peppermint tea products, displayed as overlay on a scale of 1:1 and showing 'fingerprints' of volatile compounds without determining the single compounds. The figure reveals a generally lower abundance of analytes in tea of company 1, although all characteristic compounds appear on both sides, indicating both teas being dried plant material of the same species but with different qualities.

Medicinal and dietary use of medicinal plants

Biologically active plant metabolites are eligible for use as pharmaceuticals or as food/feed supplements (DEWICK, 2009; FRANZ, 2009). While food/feed additives intend to supplement normal nutrition, pharmaceuticals heal or prevent diseases or ailments or fend off pathogens. Advisory expert boards of federal ministries, e.g. AGES (Austrian Agency for Health and Food Safety Ltd.), do the differentiation in Austria and BfArM (Federal Institute for Drugs and Medical Devices) in Germany.

Medicinal use

In human and animal herbal medicines, standardisation due to varying composition of compounds is difficult. Mostly a range of organic chemicals or a certain ratio, like the ratio of α-bisabolol: bisabolol-oxides in German chamomile, is the desired criterion for medicinal use. These criteria might be defined by the pharmacopoeia (e.g. The European Pharmacopoeia, Ph. Eur.) or by the producing company (FAEHNRICH et al., 2014).
A borderline case of secondary metabolites as feed or as medicine is an often-observed fact, that herbivorous animals, if they can select their fodder and are led by the taste, which is directly linked to the secondary metabolites, choose plants effective against possible infections or parasitic infestations. This observation is known as ability for 'self-medication' (VILLALBA et al., 2010).

Use in feed supplementation

In this review, focus lays on use of secondary plant metabolites in feed additives, which in some cases may have similar effects as in dietary food additives for humans, but are less investigated (FAEHNRICH et al., 2016a). Phytogenic feed additives can be fed as plant products, including essential oils and extracts (FRANZ, 2009). Allocation of feed additives to categories and functional groups according to their properties and functions are determined in the Commission Regulations of the European Union (No. 1831/2003 and Annex 2019/962). In this review, again, the three main groups: terpenes, polyphenols, and alkaloids serve as classification.

Terpenes in feed supplementation

Terpenes mostly influence the taste of feed. Therefore, the palatability of feed, the appetite, feed intake and the performance of farm animals can be altered by occurrence of terpenes in feed. Terpene-based feed additives often are considered as substitutes for the legally banned antibiotics as promoters of animal performance (ABBAS et al., 2017). Following their fate in the animal's digestion, terpenes turn out to be rather stable compounds that reappear in animal products (FAEHNRICH et al., 2017). In this context, Tab. 2 shows a range of phytogenic carotenoids in animal nutrition. The yellow to red pigmenting molecules intensify and modify colour of flesh, skin, plumage, and yolk (FAEHNRICH et al., 2016a).
Nevertheless, there could be an effect of habituation to altered feeding with terpenoid feed additives, due to a partial metabolism of terpenoid substances by enzymatic processes in the intestinal tract (DISTEL and VILLALBA, 2018). Investigations of volatile substances

Tab. 2: Excerpt of scientific documentation of carotenoid use in animal nutrition.[a]

Carotenoid source	Animal category	Intended effect	Source
Red pepper (*Capsicum* sp.)	Goldfish, Koi carp	Increase in red color intensity	HANCZ et al., 2003
Plant-derived carotenoids	Birds and fish	Coloring egg yolks and fish flesh	DAVIES, 2004
Annatto seed (*Bixa orellana*)	Laying hens	Increase in yolk pigmentation	DE CARVALHO et al., 2009
Yellow corn (*Zea mays*) Alfalfa (*Medicago sativa*) Marigold (*Tagetes erecta*) Red pepper (*Capsicum* sp.) Diverse algae (*Haematococcus pluvialis*, *Chlorella vulgaris*, a.o.)	Fish	Enhancement of reproduction performance, anti-oxidative capacity, provision of bright red, yellow and orange coloration of skin or flesh, enhancement of consumer acceptability	GARCIACHAVARRIA and LARAFLORES, 2013
Marigold (*Tagetes erecta*)	Poultry	Increase of feed intake, egg production, yolk color; decrease of egg cholesterol, feed conversion	NURAINI and DJULARDI, 2017

[a] excluding animal experiment models for human medicine
(Source: FAEHNRICH et al., 2016a, modified)

in raw milk showed fluctuating amounts during a test phase. Higher amounts at the beginning of a test phase in milk of an essential oil fed group of cows vs. an untreated control group, and an approach of both groups within three weeks support this hypothesis (FAEHNRICH et al., 2017; Fig. 12)

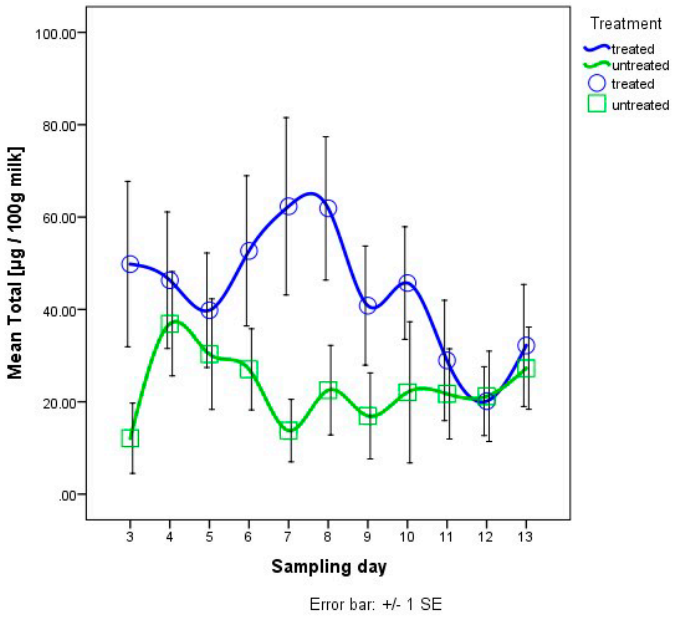

Fig. 12: Progressive diagram of total amounts of six monitored volatile substances in raw milk of the early lactation period of Simmental cows. Treated group was fed with an essential oil-bearing additive (Source: FAEHNRICH et al., 2017, modified).

Polyphenols in feed supplementation

All phenolic compounds have a broad biological spectrum of effectivity, which entails use in animal nutrition and as feed additives (except lignin, which cannot be digested and therefore, effects related to the included polyphenols, cannot be used in animal nutrition) (GRUBER, 2010). Use pursues diverse objectives:

(i) There is the colouring aspect: phytogenic, phenolic pigments, in particular yellow colouring flavones, chalcones, aurones and flavonols, the red to blue colouring anthocyanidines and the dark condensed tannins, may play a role in feed by attracting especially visually oriented animals, like poultry, and therefore, increase feed intake. However, the variability of phenolic colour expression due to intrinsic (glycosylation, oxygenation, complexation, etc) and external factors (pH, oxygen, co-pigments, etc.) limits the reasonability of use (VAN WYK et al., 2015; FAEHNRICH et al., 2016a).

(ii) The astringent taste of many phenols can foster saliva excretion; enhance appetite and palatability of feed (HOLM and HERBST, 2001; FAEHNRICH et al., 2016a). Moreover, there are a few findings elucidating an ability of animals to select phenol-rich (and therefore, anti-septic, anti-diarrheal and anti-microbial) feed in case of parasitic infection or disease (VILLALBA et al., 2010; DISTEL and VILLALBA, 2018).

(iii) The microbiome of the animals' gut (both, monogastric and ruminant animals) might be shifted in a positive way due to the antimicrobial activity of polyphenols, e.g. resulting in a favourable fatty acid composition and energy output (milk production instead of methane production) in ruminants (BRENES et al., 2016).

(iv) The protein-modulating properties of phenols, due to their hydroxyl groups and their ability to bind with proteins in different ways (hydrogen bonds, ionic and covalent bonds) can explain the above-mentioned antimicrobial effects. The more OH-groups (e.g. in condensed and hydrolysable tannins), the stronger is the effect (VAN WYK et al., 2015). This binding ability can in case of monogastrics, exert an anti-nutritive effect, as protein digestibility may be decreased. In ruminants, the same effect might enhance the forwarding of bound – and therefore undegraded – proteins from the rumen to a later stage of digestion. In ruminants, this effect can help to maintain an optimal ruminal pH (BRENES et al., 2016; FAEHNRICH et al., 2016a).

(v) The anti-oxidative effect of phenolic compounds, due to their radical scavenging ability, is another health promoting property. This ability derives from the huge number of hydroxyl groups, which can donate hydrogen atoms to free oxygen-radicals, thereby terminating oxidation of lipids and other molecules (BRENES et al., 2016). In particular in stress situations, e.g. cows in the peri-parturition phase, high environmental temperature, feed contaminated with fungal toxins, etc., when reactive oxygen species (ROS) might appear, health of the animal can be supported by phenolic feed additives (MA et al., 2019).

(vi) Phenols can intercalate with DNA and suppress signalling pathways of inflammation transcription factors, e.g. NF-κB, thereby decreasing intrinsic inflammation response and saving energy for growth and performance. This immunological effect may be a reason to discuss polyphenols (like also some alkaloids in small amounts; KHADEM et al., 2014) as potent substitutes for the legally banned application of antibiotics as growth promoters (VAN WYK et al., 2015; CHEN et al., 2018).

(vii) An often pursued effect of polyphenolic feed supplementation is the enhanced product quality of the animals' product, such as meat, egg or milk. Using the anti-oxidative capacity of polyphenols, the lipid oxidation will be attenuated, the oxidative stability enhanced and the shelf life of the product will be extended (BRENES et al., 2016; FAEHNRICH et al. 2016a). Moreover, phenolic supplementation can decrease cholesterol content in the egg yolk (LAUDADIO et al., 2015).

(viii) A further, mostly undesired effect of specific phenols, namely of isoflavones, secondary plant metabolites of legumes (particularly of the subfamily Papilionideae), is due to similarity with the mammalian female hormone oestrogen. Phytogenic isoflavones, for this reason also called 'phytohormones', cause hormonal effects in female animals which can disturb the reproductive cycle and fertility of female farm animals (VAN WYK et al., 2015).

However, there is a lack on exact knowledge, how and to which extent degradation of polyphenols in the course of digestive processes takes place. In general, they are assumed prone to intensive metabolism (GUTZEIT and LUDWIG-MÜLLER, 2014). Furthermore, the recovery of polyphenols in the animals' tissues depends on variability and complexing/binding of phenols, on the animal species, on the respective way of digestion, on the accompanying main feedstuff and on environmental conditions. Therefore, predictability and grade of effectivity of polyphenols as feed supplementation is limited (BRENES et al., 2016; FAEHNRICH et al, 2016a).

Alkaloids in feed supplementation

Alkaloids are generally known to be poisonous, e.g. neurotoxic (indolizidine alkaloids), paralytic (piperidine alkaloids), hepatoxic (pyrrolizidine alkaloids) or anticholinergic and anti-muscarinic (tropane alkaloids) (DIAZ, 2015). In recent studies, intensive efforts have been made to detect particularly pyrrolizidine alkaloids in food and feed (PICRON et al., 2018).

Still, there are a couple of examples of their use in nutritional supplements and feed additives. Mainly by inhibiting activation of the DNA transcription factor NF-κB and thereby avoiding costly inflammation response in mammalians, alkaloids may support the health and foster

growth and performance of farm animals (Ni et al., 2016; Faehnrich et al., 2019c). Other desired effects rely on selective anti-microbial activity of alkaloids. By shifting the bacterial microbiome in the digestive tract of ruminants, CH_4 emissions can be reduced, and the energetic performance efficiency can be increased (Dos Santos et al., 2013).

In companion animals, the health maintaining effects of isoquinoline alkaloid preparations are highly appreciated. It has been shown that prophylactic administration of isoquinoline alkaloids can work towards a ready-to-react immune system in healthy animals by maintaining all immunological values of immunoglobulins and categories of leucocytes and phenotypes of lymphocytes, and at the same time, providing anti-inflammatory properties as described above in case of inflammation (Faehnrich et al., 2018).

Conclusions and prospects

Current trends in breeding medicinal plants/production of secondary metabolites

For a long time, conventional breeding (including tissue culture and induced mutations by chemicals or radiant exposure) was the predominant form of breeding in medicinal plants. Nowadays, also biotechnological approaches to create fingerprints of genotypes and to explore relationships of species are established (Pank and Blüthner, 2009; Otto et al., 2017). Germplasm conservation and *in-vitro* propagation are seen as a possibility to avoid over-collecting from the wild and loss of genetic resources (Pérez-Garcia et al., 2006).

Still, as medicinal plants or their products are deemed natural products and the additional yield that can be achieved is marginal while establishing costs are high, genetic engineering and the generation of transgenic plants is not (yet) in the focus due to missing public acceptance – at least not as a mass production (Pank and Blüthner, 2009). Nevertheless, several researchers aim to improve secondary metabolites gain by genetic engineering of the grown plant (Abbas et al., 2017; Wang et al., 2017).

Known for almost hundred years, secondary metabolites of fungi, like of *Penicillium* spp., serves as medicine. Current research intends to increase production of anti-microbial active compounds by bacteria, fungi and plants through treatment with the metabolism modifying agents, e.g. butyrate or jasmonates (Majdi et al., 2015; Zutz et al., 2017).

Metabolic engineering approaches intend to establish and enhance microbial production of secondary plant metabolites, using e.g. genetically modified *Escherichia coli* as production host and producing e.g. the anti-oxidant polyphenol rosmarinic acid or the anti-cancer diterpenoid carnosol (originally in extracts of *Rosmarinus officinalis)* as target compounds. Such production of pharmaceutical secondary metabolites via genetically modified microorganisms is already established in a large scale (Wasternack and Strnad, 2019). This production way avoids problems with varying and mixed composition of biologically active plant compounds in the original plant extracts (Kallscheuer et al., 2018).

Current trends and approaches in analytics and effect evaluation of medicinal plant material

Drug and phyto-chemistry always seeks to refine and improve technology to overcome the challenges of varying composition and organic matrices. Recent technological advances elaborate detection and quantification methods for every single analyte, including hyphenated techniques and specific definition of stationary phase and eluent in chromatography (Gomes et al., 2018; Picron et al., 2018).

Nevertheless, due to the varying natural source material (varying in genetics, in edaphic, geographic and climatic conditions, in ontogenetic and morphological characteristics, etc.), neither an instantaneous chemical composition profile nor the genetic fingerprint of the plant provide a guarantee for the medicinal and/or health promoting effect (Van Wyk et al., 2015). Often even the mechanism of effect is not clear or the opinion concerning the effective compounds varies (Armbrüster et al., 2009; Schilcher, 2009). On the other hand, just evaluating accessions of medicinal plants/drugs, solely according to their effect, will not give sufficient information (Montanari et al., 2016). In particular, because the *in vivo* effect on the target organism will always also depend on its inherent individuality and the specific ambient conditions (Faehnrich et al., 2019a).

Therefore, a holistic approach of systems biology, including genetic disposition and expression, phytochemical evaluation of metabolites, *in-vitro*, and *in-vivo* tests with multivariate evaluation seems to be a reasonable vision for the future (Verpoorte et al., 2005).

Current trends in use and application of medicinal plants/secondary metabolites

Besides the huge field of benefits from secondary plant metabolites in human and animal nutrition, for herbal medicines and other established purposes, direct relevance in crop production shall be mentioned as an upcoming field of research (Faehnrich et al., 2017). Intercropping systems can e.g. increase the polyphenol content of the cropping partners. As outlined by Salehi et al. (2018), buckwheat increases the anti-oxidative capacity after intercropping with fenugreek. Interactions of crops and their environment, e.g. by mycorrhiza colonization or use of bio-fertilizers in organic farming systems show greatest positive influence on secondary metabolism (Ahmadabadi et al., 2018).

Besides the pharmaceutical value, the phytosanitary effect of increased secondary compound content in crops is of great interest to enhance their self-defence against pathogens, herbivores and diseases and thereby decrease the need of measures of plant protection, in particular of chemical pesticides (Salehi et al., 2018). As defence is one of the main natural tasks of secondary metabolites, they will also have the potential to protect other crops directly or by enhancing resistance reactions within the plant's metabolism (Abbas et al., 2017; Cohen et al., 2016). Therefore, the implementation in crop protection might result in the use of plant extracts and/or whole aromatic/medicinal plants as bio-stimulants or as bio-pesticides (Nollet and Rathore, 2015; Schuster and Schmitt, 2015).

Moreover, allelopathic, weed suppressing effects of secondary plant metabolites can provide benefits in farming systems (Abbas et al., 2017). Allelopathic effects can occur in rotational sequences, e.g. when secondary metabolites bearing plants are left as mulch or are applied as a soil amendment (Macias et al., 1998; Ferguson et al., 2009). Often medicinal and/or invasive plant species have allelopathic features (Abgrall et al., 2018).

Still, in the fields of incorporating secondary metabolites/medicinal plants in crop management a lot of research is needed to define effective compounds or compositions in changing environments, without causing phytotoxicity to the target crop. Moreover, balancing the efficacy and application costs and the expected gain in crop production might be the challenge for market success (Olany and Larkin, 2006; Pino et al., 2013).

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Conflict of interest

No potential conflict of interest was reported by the authors.

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