Nat. Volatiles & Essent. Oils, 2017: 4(4): 1-13

NVEO
Natural Volatiles & Essential Oils

REVIEW

Biological properties of essential oils and volatiles: Sources of variability

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Abstract

Essential oils are a specific type of extracts that result either from hydro-, steam- or dry-distillation from any part of a duly identified botanical species, or by a mechanical process without heating, called expression, from the epicarp of *Citrus* fruit species. Any other type of extraction may provide a more, or less, volatiles-rich extract, but it is not an essential oil. Essential oils, per se, do not exist in the plant, since they result from a specific extraction procedure. In the plant there is a wide array of metabolites, only some of which will be extracted in the form of an essential oil, with variable composition even if just considering that they can either be obtained by distillation or expression. Man has used for long, both volatiles and essential oils, for a wide range of purposes in the pharmaceutical, food, beverage, cosmetic and perfumery industries, among others. The described biological activities of volatiles and essential oils are wide, but the results are not always congruent. Awareness of the factors that may influence the essential oil chemical variability and ultimately its biological activity, efficacy and safety, are thus very important. These comprise, a) the knowledge on the difference between plant volatiles and essential oils, b) the avoidance of botanical misidentification, c) getting information on plant parts chemical composition variability, the existence of chemotypes and essential oil components enantiomers, and also f) exploring different biological activity assay conditions, which was reviewed in this present work.

Keywords: Essential oils, volatiles, biological properties, chemical composition

Introduction

Plants produce and/or accumulate a diverse assortment of metabolites that are generally defined as primary metabolites or secondary metabolites (Figueiredo & Barroso 2015). Volatiles are among the diverse constituents that plants produce and accumulate in specific secretory structures. All land plants, either bryophytes (liverworts, hornworts and mosses), or tracheophytes (vascular plants) produce volatiles.

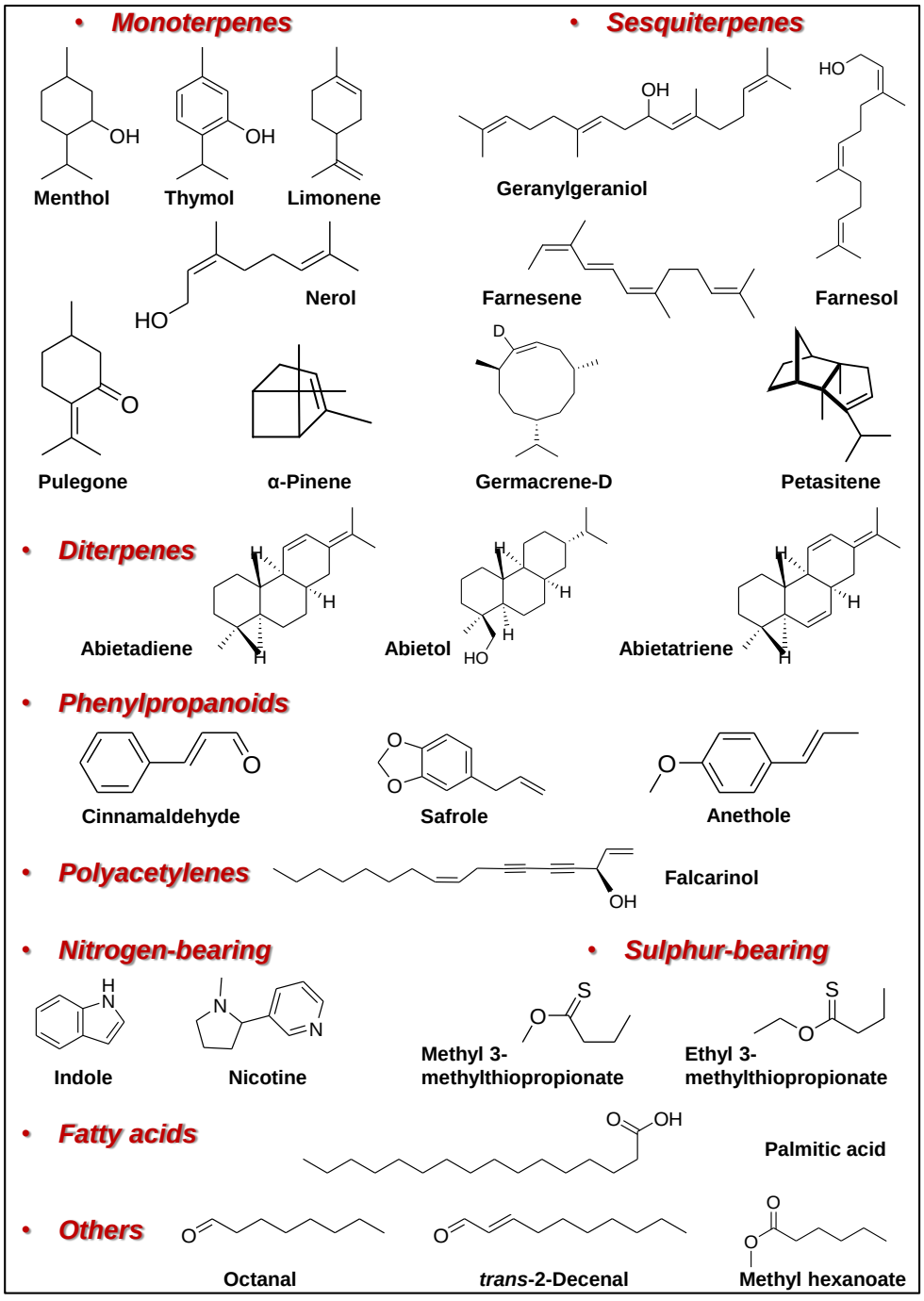
Plant volatiles correspond to (a) a complex mixture of compounds which have the property of naturally volatilizing under appropriate environment temperature and humidity conditions, allowing, for example, that we may smell the aroma of a rose, or of jasmine, or (b) include an equally complex mixture of compounds that are extracted from plants by different methodologies at home, laboratory or industrial level.

These compounds can be composed only by carbon and hydrogen, or additionally have oxygen, nitrogen or sulphur in their molecules. In general, these compounds fall down in the chemical classes of terpenes, phenols, nitrogen-bearing compounds and other primary and/or secondary metabolites derived from diverse metabolic pathways (Figure 1). However, these substances are often present in the plant at reduced levels and/or different ratios which are species specific.

Plants use volatiles as a part of their strategy to adapt to the environmental biotic and abiotic factors (Figueiredo and Barroso 2015), and man has learned to use both volatiles and essential oils for their diverse array of properties. In addition to the well-known therapeutic actions and positive effect on health and well-being, volatiles and essential oils find application in other fields such as food, beverages, cleaning, textile,

perfume and cosmetic industries, to name a few. Likewise, many researchers devoted their studies to very broad subjects within this field of research.

Figure 1. Examples of volatile constituents isolated from plants.



In the great majority of the cases it is impossible to differentiate in an essential oil its diverse biological activities, and so its action, in a certain circumstance, is the result of the interaction of several of its components properties and, many times, of the way and amount used. For instance, essential oils may mitigate anticancer activity not because they act on the cancer itself but because of their antioxidant activity they lower the toxic effects of free radicals induced by several cancers, or even from anticancer agents.

But being bioproducts, essential oils are also prone to variability, thus requiring assurance of constancy and quality, to guarantee efficacy and safety. On the other hand, the use of essential oils is not devoid of

undesired side effects which are, in most cases, related with their misuse. Knowledge on the essential oil chemical composition is essential for understanding possible tolerance mechanisms and/or adverse effects.

The knowledge on the biological properties of volatiles and essential oils is important, because they combine being a) natural and biodegradable, b) less toxic than chemical pesticides, and usually showing low toxicity to mammals, if accepted mean daily doses are respected, c) able to accomplish, simultaneously, the function of more than one of their synthetic equivalents and d) less expensive than some drugs.

The reported biological activities of volatiles and essential oils are immense and to classify them is not an easy task. For simplicity, in Table 1 they are separated into therapeutic and non-therapeutic. Nevertheless, the properties intermingle, and many of the therapeutic uses support the non-therapeutic ones. For instance, essential oils and volatiles may have an external use as antiseptic and disinfectant, but they can be used in household products exactly for the same properties. On the other hand, whereas many studies focus on the Human or animal use, some studies also deal with a more ecological perspective, viewing to understand the importance of volatiles in plant-abiotic and plant-biotic relations.

Table 1. Some of the reported biological properties of volatiles, essential oils and their constituents.

Therapeutic
External: Antiphlogistic / anti-inflammatory, antiseptic and disinfectant, deodorant, hyperemic, among others.
Internal: Analgesic / antinociceptive, antibacterial, anticancer, antioxidant, antiphlogistic / anti-inflammatory, anti-rheumatic, antispasmodic, antiviral, carminative, expectorant, hyperemic, penetration enhancer, sedative, among others.
Non-therapeutic
Antiseptic and disinfectant
Absorption enhancer
Preservative and spice
Repellent
Pesticide
Toxic
Defence and attraction in plant-abiotic and plant-biotic relations

A very large number of studies have been performed on this wide range of biological properties, and trying to summarize the gathered data is complex, because of the diversity of testing conditions used (some using commercial essential oils, others essential oils isolated from plants from the wild, cultivated plants, different plant parts, different extraction methods) and because, in some cases, results are different, or even contradictory.

In view of this large amount of data, and results variability, this review will try to pinpoint some relevant aspects regarding the reasons for this variability, with some examples.

Plant volatiles *versus* essential oils

The isolation of plants active ingredients, at industrial or at laboratory level, may be carried out by different extraction methodologies, depending on the ultimate goal, using, among others: i) organic solvents, ii) supercritical fluids, iii) fats, iv) mechanical means, with or without temperature and solvent, v) specific selective procedures, and vi) distillation or expression (Figueiredo et al. 2014).

Whereas these different methods can be used to extract volatile compounds from plants, only two are used to obtain an essential oil (Council of Europe 2010): a) hydro-, steam- or dry-distillation from properly identified botanical species or from its different parts, or b) a mechanical process without heating, termed expression, in the case of epicarp of *Citrus* fruit species, such as orange, lemon, mandarin, grapefruit, among others.

It is noteworthy that an essential oil as such, with its characteristic chemical composition, does not exist in the plant, and it is a product of a suitable extraction process. In the plant there is a complex mixture of compounds, some of which are released naturally into the atmosphere, others may be extracted by distillation or expression, and there are others that are non-volatile and, as such, require other specific extraction methodologies. Thus, any phytochemicals extraction process will always give a partial perspective of the whole of the components that exist in the plant and the extraction methodology used should always take into account the characteristics of the type of compound(s) to be isolated and the goal of the study.

Nevertheless, many times either in the literature or in commercial samples, the extracts are called, or the labelling mentions, an essential oil, when the extraction procedure used does not provide an essential oil, with obvious implications in the chemical composition and biological activity of the extract.

Table 2. Main components (≥5%) of commercial available labelled *Helichrysum italicum* essential oils (Hi 12 and Hi 13) and of published data (adapted from Ventura & Lima, 2017).

	Helichrysum italicum				
	Hi 12*	**Hi 13***		**Essential Oils**	
Components	**EINECS 289918-2**		**Corsica (France)**	**Italy**	**Portugal**
			Bianchini *et al*. 2001	Satta *et al*. 1999	Costa *et al.* 2015
α-Pinene	t	18.8	0.7-2.9	0.1-0.2	53.5
Limonene		1.2	1.9-7.5	1.2	0.5
Linalool		t	1.0-2.8	9.1-14.9	0.1
4,6-Dimethyloctan-3,5-dione			1.0-11.3		
Nerol		0.9	2.0-4.9	nd-10.7	
Neryl acetate	11.6	11.4	15.8-42.5	nd-28.9	
Italicene *	2.7	9.9	0.9-3.4	2.3-4.2	1.2
4,6,9-Trimethyldec-8-en-3,5-dione			0.8-5.6		
Neryl propionate			1.6-6.7		
ar-Curcumene	21.7	8.1	0.8-4.6	4.5-4.8	2.8
γ-Curcumene		8.1	0.8-12.9	11.4-18.2	27.4
δ-Cadinene				0.8-5.6	0.3
Eudesm-5-en-11-ol			nd-5.1		
Rosifoliol				3.9-20.2	

* Commercial available labelled *Helichrysum italicum* essential oils from 2012 (Hi 12) and 2013 (Hi 13), EINECS 289918-2: *Helichrysum* leaf absolute CAS 8023-95-8 CoE225, nd: not detected, t: traces (<0.05%).

As an example, Ventura & Lima (2017) compared the chemical composition of commercial available labelled essential oils from *Helichrysum italicum*, with published data on the chemical composition of the essential oil from this species collected at different locations in the Mediterranean area, Table 2. Not only it is clear that the chemical composition varies with provenance, probably related to the subspecies used, the distillation length, plant developmental stage, plant part used, among other factors, but there is also a clear difference between the reported essential oils composition and that from the commercial samples. Indeed, although available as an essential oil, the label of the commercial samples also mentioned EINECS 289918-2. The presence of this code from the European Inventory of Existing Commercial Substances (EINECS) means that instead of being an essential oil, it was an absolute of *Helichrysum*, which partly explains the difference in chemical composition, which would have also an implication on the biological properties. So, when evaluating samples from commercial origin, it is important to certify that an essential oil is being used, or at least to state the correct type of extract used in the evaluation.

Plant quality control

Botanical misidentification is still a major problem, particularly when using wild collection of plant material and, sometimes, solely based on local common names. Accordingly, this will have obvious implications in the chemical composition as well as on the biological property of the isolated essential oil or of any other type of extract.

Sullivan (2009) performed a comparative analysis of in-laboratory-isolated essential oils from commercially available herbal products of *Eucalyptus globulus* (eucalyptus), *Foeniculum vulgare* (fennel) and *Mentha x piperita* (peppermint), with the commercial essential oils counterparts, used in aromatherapy practice. Whereas only one out of the nine commercial *E. globulus* essential oils analysed showed some differences in the chemical composition, and some differences were found for essential oils isolated from *F. vulgare* probably because of using bitter instead of sweet fennel, the most striking discrepancy was found with the samples from *M. piperita*, Table 3.

Table 3. Minimum and maximum percentage range of main components (≥5%) from the essential oils isolated from *M. piperita* labelled herbal product, from commercial essential oils, and reference data from the Portuguese Pharmacopoeia (FP) and the International Organization for Standardization (ISO) (adapted from Sullivan, 2009).

Components	***Mentha x piperita* essential oil main components (≥5%)**							
	Commercial				**Specifications**			
	Herbal product		**Essential oils**		**FP (2005)**		**ISO (2006)***	
	Min	Max	Min	Max	Min	Max	Min	Max
1,8-Cineole	7.1	7.5	1.8	4.8	3.5	14.0	3.0	8.0
Limonene	6.0	7.1	1.8	4.8	1.0	5.0	1.0	3.0
Menthone	0.1	0.1	22.0	26.8	14.0	32.0	13.0	28.0
Isomenthone	0.3	0.3	3.1	5.7	1.5	10.0	2.0	8.0
Neomenthol	t	t	3.2	4.8			2.0	4.5
Menthol	t	t	36.4	53.7	30.0	55.0	32.0	49.0
Carvone	42.1	43.4	t	t		1.0	0.5	3.0
Pulegone	21.1	21.7	0.4	2.4		4.0		

FP: Farmacopeia Portuguesa (2005), ISO (2006): ISO 856:2006, * several types, t: traces (<0.05%).

The commercial essential oil samples of *M. piperita* analysed were all found to be within the assessment of quality specifications of the Portuguese Pharmacopoeia (Farmacopeia Portuguesa, 2005) and of the International Organization for Standardization ISO 856:2006, with menthol and menthone as dominant compounds. The chemical components cluster analysis from the five commercial essential oil samples analysed showed a very good level of correlation between them. They were, however, uncorrelated with those from the essential oils isolated from the commercial herbal product due to incorrect labelling and species identification. The analysis of the essential oil isolated from the commercial herbal product revealed carvone and pulegone as main components, suggesting that the herbal product was *M. pulegium* (pennyroyal) instead of *M. piperita*.

The use of just common names, without a proper botanical certification, is also a major cause of plant misidentification and commercialization, both as herbal products and even in crop seeds. Common names often refer to different species, which, sometimes, do not even belong to the same plant family, Table 4.

Table 4. Examples of some Portuguese plant common names and the plant species to which they may refer to.

Portuguese common name	**May refer to, according the region of Portugal or abroad**	**Family**
"Amieiro"	*Alnus glutinosa*	Betulaceae
	Frangula alnus	Rhamnaceae
	Populus alba	Salicaceae
"Carqueja"	*Pterospartum tridentatum* (Portugal mainland)	Fabaceae / Leguminosae
	Ulex europaeus (Portugal, Madeira island)	Fabaceae / Leguminosae
	*Baccharis trimera (*Brazil*)*	Asteraceae
"Cedro"	*Cedrus* spp.	Pinaceae
	Thuja spp.	Cupressaceae
"Erva-cidreira"	*Cymbopogon citratus*	Poaceae
	Melissa officinalis	Lamiaceae / Labiateae
"Loendro"	*Nerium oleander*	Apocynaceae
	Rhododendron ponticum	Ericaceae
"Macela"	*Chamaemelum* spp.*, Matricaria* spp.*,* and even *Helichrysum* spp.	Asteraceae / Compositae
"Perpétua-das-areias"	*Helichrysum italicum* and *Helichrysum stoechas*	Asteraceae / Compositae
"Rosmaninho"	*Lavandula* spp. and *Rosmarinus officinalis*	Lamiaceae / Labiateae

"Carqueja" is a highly appreciated plant in Portugal, both for culinary uses and because the infusion of the flowers is used for therapeutic properties (Grosso et al., 2007). Nevertheless, in the mainland Portugal "carqueja" refers to a *Pterospartum* species, whereas in the Madeira archipelago it refers to an *Ulex* species, and in Brazil, the same common name refers to a *Baccharis* species from a different family, Table 4. Recalling the previous example of *H. italicum*, both this species and *H. stoechas*, are known in Portugal by the same common name, "perpétua-das-areias" (everlasting). This may constitute an additional factor of chemical variability between plant extracts.

These examples stress the importance of a correct plant identification, both at the academic, and at the essential oil producer level, as this will determine the chemical composition of the final product.

Chemical composition, chemotypes and enantiomers

Phytochemcials in general, and volatiles or essential oils in particular, can be extracted from different plant parts, namely bark, flowers and flower parts, fruits, leaves, petioles, roots or rhizomes, seeds and/or stems. It is well known that the plant part can, in many cases, play a major role in the final composition of the essential oil (Figueiredo et al., 2008) as well as on its advised use (Ruppert-Aulabaugh, 2014). This different composition is many times related not only with the defensive or attractive role of the compounds in the plant, but also to where and how they are accumulated (Figueiredo & Barroso 2015)

Plant metabolites in general are usually accumulated either in the vacuole, in plastids, in the cell wall, in the cuticle, and/or in specialized glandular structures (Figueiredo & Barroso 2015). Nevertheless, specialized glandular structures do not always have a regular distribution over the plant (Figueiredo et al., 2008), which, on itself, may determine different chemical profiles upon isolation. In addition, for instance metabolites accumulated in vacuole, are usually not volatile because they are accumulated in a soluble glycosylated form. To extract the volatile part of these molecules the glyosidic bound has to be broken prior to volatiles isolation (Belhattab et al. 2005).

Ruppert-Aulabaugh (2014) reported two well known cases of essential oils isolated from the same plant, with different chemical profiles and different uses in aromatherapy. In one case the reason for this difference is the plant part used and in other the methodology of essential oil isolation used. *Angelica archangelica*

provides a root essential oil and a seed essential oil. Whereas the seed essential oil is not considered a photosensitizer, the root essential oil may be, thus additional cautions should be taken on its use. Whereas the essential oil obtained, without heating, by the mechanical process of expression from the external part of the pericarp of *Citrus* × *paradisi* fruit is known to be photosensitizing, the distilled version is considered non-phototoxic.

In addition to the variable chemical composition due to the use of different plant parts or different isolation procedures, the presence of chemotypes must also be evaluated for its influence in the biological activity of the essential oil.

Chemotypes are chemically distinct groups within a species, that is, plants of the same species, which being phenotypically similar, differ in the type, or proportion, of their chemical constituents.

The essential oils of several species such as *Hypericum* spp. (St. John's wort), *Lavandula* spp. (lavender), *Mentha* spp. (mint), *Ocimum* spp. (basil), *Origanum* spp. (oregano), *Rosmarinus* spp. (rosemary), *Salvia* spp. (salvia), or *Thymus* spp. (thyme), show several chemotypes. Althought not all essential oils show chemotypes, and only some chemotypes are available on the market, it is important to stress that different chemotypes may show diverse performance to a biological property, as shown with the example of *Thymus caespititius* essential oils chemotypes, Table 5.

T. caespititius essential oils show four different chemotypes, carvacrol, sabinene, thymol and α-terpineol, although sabinene chemotype is much less frequent than the others (Figueiredo et al. 2008a). Dandlen et al. (2011), showed that from the three most common chemotypes, in general, the lowest acetylcholinesterase (AChE) inhibition activity was found with α-terpineol rich essential oils, Table 5.

T. caespititius thymol and carvacrol rich essential oils were the most effective in AChE inhibition, although it is also clear from Table 5 that there are thymol and carvacrol rich essential oils which are less effective. This clearly shows that the effectiveness of an essential oil relies on the essential components as a whole and not just on its isolated constituents due to the existence of synergistic and antagonistic effects between its components. Data from Table 5 also shows that it is important to evaluate several samples, because the chemical composition will influence the final biological activity. Other studies (Faria et al. 2013), in which the essential oils were fractionated to evaluate the separate contribution of the fractions containing hydrocarbons or oxygen-containing molecules also highlighted distinct synergistic or antagonistic interactions between the essential oil components, influencing the overall activity.

Table 5. Harvesting place, four main components and acetylcholinesterase (AChE) inhibition ability of *Thymus caespititius* essential oils, chemotypes carvacrol, α-terpineol and thymol (adapted from Dandlen et al., 2011).

Thymus caespititius Harvesting place	Essential Oil Four Main Components (%)	AChE inhibition (IC_{50} μg/ml)*
Caramulo (MP)	α-Terpineol 40, p-cymene 14, γ-terpinene 5, τ-cadinol 5	5898.0 ±235.4
Covide (MP)	α-Terpineol 35, p-cymene 17, γ-terpinene 9, τ-cadinol 6	4647.2 ±26.1
Lordelo (MP)	α-Terpineol 24, p-cymene 16, γ-terpinene 12, τ-cadinol 7	1766.5 ±18.3
Óbidos (MP)	α-Terpineol 52, p-cymene 15, γ-terpinene 7, τ-cadinol 6	12448.0 ±517.6
Outeiro (MP)	α-Terpineol 41, p-cymene 14, γ-terpinene 9, β-caryophyllene 3	3000.6 ±125.3
Pico (A)	Carvacrol 51, carvacryl acetate 19, p-cymene 6, γ-terpinene 4	567.4 ±12.8
Pico Verde (A)	Carvacrol 32, thymol 23, carvacryl acetate 7, p-cymene 6	181.4 ±4.2
Planalto Central (A)	Carvacrol 62, carvacryl acetate 12, α-terpineol 3, p-cymene 3	158.9 ±2.4
Ponta dos Rosais (A)	Thymol 25, α-terpineol 19, p-cymene 12, γ-terpinene 10	3601.6 ±13.4
Serra do Cume (A)	Thymol 35, carvacrol 13, p-cymene 8, thymyl acetate 8	139.1 ±2.5
Terras de Bouro (MP)	α-Terpineol 24, γ-terpinene 14, p-cymene 12, γ-eudesmol 6	2579.1 ±2.3
Vilarinho das Furnas (MP)	α-Terpineol 42, p-cymene 14, γ-terpinene 6, β-caryophyllene 4	4219.5 ±37.2

MP: Mainland Portugal. A: Azores archipelago, Portugal. * Concentration providing 50 % inhibition.

One other important point, often neglected, that contributes to chemical and biological activity variability is the chirality of essential oil components, when existing. Different enantiomers of chiral compounds often taste and smell differently and have different biological effects (Stahl-Biskup, 2002).

Studies performed on the enantiomeric distribution of the chiral compounds present in high relative amount in *T. caespititius* essential oils, such as sabinene, terpinen-4-ol and α-terpineol (Pereira et al., 2000), or on the enantiomeric ratio of the monoterpene hydrocarbons α-pinene, camphene, sabinene, β-pinene, limonene and of the oxygen-containing terpinen-4-ol, α-terpineol and borneol in *T. camphoratus* essential oils (Miguel et al., 2009), showed clear enantiomeric polymorphism. Further enantioselective evaluation of essential oils may be of relevance in understanding the different biological performance of essential oils that show similar chemical composition.

Biological activity assay conditions

There is a large number of factors that additionally may affect the final biological activity of one essential oil, at and during the assay conditions, Table 6.

Table 6. Variability in biological activity assay conditions.

Variability in assay conditions
Different test types for one type of biological property evaluation
For some biological properties, the diversity in organisms, strains or cell types to be evaluated
Dose
Way of application
Moment of application
Comparison with control substances
Toxicological tests
Direct contact assays do not necessarily reproduce the response in real environment

Not only can one essential oil be evaluated for the diverse reported biological properties, Table 1, but also each of these biological activities can be assessed in different ways. Considering any one of the reported biological properties of volatiles, essential oils and their constituents, there are always diverse ways of evaluating it, each of which will evaluate a particular characteristic performance, will deal differently with the essential oil volatility and poor water solubility.

Table 7. Diversity of studies, and methodologies used, on the biological activity assessment of the essential oils, of different chemotypes, isolated from *Thymus caespititius*, grown in Portugal.

Thymus caespititius	**Biological activity / Assay method**	**Reference**
	Antiacetilcolinesterase	
Carvacrol, thymol and α-terpineol chemotypes	AChE inhibition	Dandlen et al. 2011a
Thymol/carvacrol type	AChE inhibition	Aazza et al. 2016
	Anti-hyperglycaemic	
Thymol/carvacrol type	Inhibition of α-amylase and α-glucosidase	Aazza et al. 2016
	Anti-inflammatory	
Thymol/carvacrol type	NO, 5-lipoxygenase assay	Aazza et al. 2016
	Antimicrobial	
Carvacrol, thymol and α-terpineol chemotypes	Growth inhibition of Helicobacter pylori (2 strains), Staphylococcus aureus, Salmonella enterica, Haemophilus influenzae, Streptococcus pneumoniae and Candida albicans (2 strains) by agar disc diffusion method and drop method for MIC	Dandlen et al. 2011
α-Terpineol type	Growth inhibition of *Aspergillus*, *Candida*, *Epidermophyton*, *Microsporum*, *Tricophyton* strains, by broth macrodilution method based on the Clinical and Laboratory Standards Institute (CLSI) reference documents M27-A3, S3, for MIC	Pinto et al. 2014
	Antioxidant	
α-Terpineol type	TBARS with and without ABTS	Miguel et al. 2004
Carvacrol, thymol and α-terpineol chemotypes	DPPH•, hydroxyl and superoxide free radicals scavenging. TBARS assay	Dandlen et al. 2010
Carvacrol, thymol and α-terpineol chemotypes	ABTS and peroxyl free radicals scavenging	Miguel et al. 2015
Thymol/carvacrol type	ABTS, ORAC, NO, Chelating, Liposomes, TBARS, 5-lipoxygenase assay	Aazza et al. 2016
	Antiproliferative / Cytotoxic	
Carvacrol type	Cell line 23132/87 DSMZ nº ACC201, human gastric adenocarcinoma cell type, determined by Tetrazolium (MTT) method	Dandlen et al. 2011
Carvacrol, thymol and α-terpineol chemotypes	THP-1 leukaemia cell line. Viability determined by Tetrazolium (MTT) colorimetric assay	Miguel et al. 2015
	Nematicide	
Carvacrol type	Direct contact assays against *Bursaphelenchus xylophilus*	Barbosa et al. 2010
Carvacrol and α-terpineol chemotypes	Direct contact assays against *Bursaphelenchus xylophilus*	Faria et al. 2013
Carvacrol, thymol and α-terpineol chemotypes	*Meloidogyne chitwoodi* hatching inhibition direct contact assays	Faria et al. 2016

ABAP: 2,2'-azobis(2-amidinopropane). ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid). AChE: Acetylcholinesterase. DPPH: 2,2-diphenyl-1-picrylhydrazyl. MIC: minimal inhibitory concentration, MLC: minimal lethal concentration. NO: Nitric oxide. ORAC: Oxygen Radical Absorbance Capacity. TBARS: Thiobarbituric acid reactive species.

If, as an example, antinociceptive / analgesic evaluation tests are considered, studies may address a) transient pain, by applying thermal, mechanical, electrical and chemical stimulation, or b) continuous pain, by inducing polyarthritis. These may involve, the abdominal writhe test, capsaicin-injection into the mouse hind paw, carrageen induced paw oedema, formalin-induced nociception, hot-plate test, pain-induced functional impairment model in the rat (PIFIR model), tail-flick test, tail immersion test, among several others.

Many times the results are not directly comparable, not all of them use controls, or test both the essential oil and essential oil constituents (Adorjan & Buchbauer, 2010; Lenardão et al., 2016).

Combining the variability of biological properties possible, Table 1, the tests available and the chemical variability, it is easy to understand the diversity of data obtainable, even for less studied species, Table 7. The lower performance, or absence of activity when using one test does not mean that a positive results is not obtained with another type of test to evaluate a certain biological property, Table 8. The same essential oil can also exhibit different capabilities toward different strains of the same microorganism, Table 9.

Table 8. Antioxidant activity of *Thymus caespititius* essential oil thymol/carvacrol type assessed by different methodologies (adapted from Aazza et al., 2016).

Antioxidant activity tests	***Thymus caes*pititius essential oil thymol/carvacrol type [IC_{50} (µg/ml)]**
ABTS	10.0 ±0.0
ORAC*	2152.6 ±16.4*
NO	300.0 ±0.1
Chelating	-
Liposomes	160.0 ±0.0
TBARS	130.0 ±0.0

ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid, ORAC: Oxygen Radical Absorbance Capacity, NO: Nitric oxide, TBARS: Thiobarbituric acid reactive species. * Micromoles Trolox equivalent (TE)/g

Table 9. Growth inhibition of two *Helicobacter pylori* strains with different samples of *Thymus zygis* essential oils (adapted from Dandlen et al., 2011).

***Thymus* species essential oil**	**Harvesting place**	**EO main component (%)**	***Helicobacter pylori* strain [Inhibition zone (mm)]**	
			J99	**26695**
T. zygis sylvestris	Alcanena	Carvacrol 32	7.7 ±2.9	15.3 ±0.6
T. zygis sylvestris	Condeixa	*p*-Cymene 36	10.0 ±0.0	18.0 ±0.0
T. zygis sylvestris	Covão do Coelho	Carvacrol 35	10.3 ±0.3	17.3 ±1.5
T. zygis sylvestris	Duas Igrejas	*p*-Cymene 39	7.3 ±2.3	18.7 ±0.6
T. zygis zygis	Rebordãos	Carvacrol 44	16.0 ±1.7	20.8 ±1.4
Chloramphenicol / Amphotericin B			36.0 ±1.0	36.3 ±0.6

Other factors are also extremely important and determinant in the variability of results observed, Table 6, such as the dose of essential oil used, the way it is applied (pure, vapour phase or dispersion, in an oily carrier, in a wetting agent, in an organic solvent, in liposomes, among others), or the moment of application (for anti-microbial activity the growth phase and developmental stage of the microorganism is important). The use of positive control substances, whenever possible, is also relevant to asses not only a comparative performance, but also to evaluate if the test is working properly. Likewise, negative controls should be assessed, namely to discard secondary activities from any type of carrier, as well as toxicological tests to determine the toxic effect on non-target organisms. Most of the times the tests are performed in direct contact assays which do not necessarily reproduce the response in real environment, where other abiotic (pH, lixiviation, light, temperature, *etc.*) or biotic (biotransformation by cells or organisms) factors may drastically change the response to the essential oil.

In conclusion, available data on the biological properties of essential oils is very broad, and, sometimes, results are not consistent. A number of factors which may be considered potential sources of this variability were addressed, namely type of extract, botanical certification, chemical polymorphism and assay conditions.

Although the use of essential oils is Generally Regarded as Safe (GRAS), care should be taken on their use, since the way of action is still many times not fully understood, and/or unspecific, that is, both target and non-target cells or organisms may be affected. In view of this, after an initial screening of the effectiveness of essential oils, a further step should be taken to assess the toxicity to non-target cells. At this level, biotechnological models (Faria et al., 2015; Faria et al., 2016) may provide an intermediary step to evaluate the lowest effective concentrations that does not harm non-target cells, or the existence of mechanisms of detoxification and/or biotransformation, by either target and non-target cells or organisms.

ACKNOWLEDGMENT

Partially funded by Fundação para a Ciência e a Tecnologia under UID/AMB/50017/2013, FEDER PT2020-Compete 2020. Part of this work was presented in ISEO 2017, Pecs, Hungary.

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