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Content

1 Abstract

This paper provides a more in-depth insight into the biological, chemical, and pharmacological profile of selected conifer essential oils. Conifer essential oils are transparent and normally colorless. Conifer essential oils do not have a specific and permanent composition, it rather depends on diverse agro-ecological conditions. They are compounds of terpenic hydrocarbons, phenylpropanoids, and their derivatives. The major compounds of conifer essential oils are pinene, limonene, β-phellandrene, ∆3-carene, and germacrene D. In this work, the essential oils of *Pinus cembra* L., *Pinus mugo* Turra, *Pinus sylvestris* L., and *Pinus nigra* J.F. Arnold are investigated while taking into consideration their components and their biological activity.

2 Zusammenfassung

Diese Arbeit bietet einen tieferen Einblick in das biologische, chemische und pharmakologische Profil ausgewählter ätherischer Nadelöle. Die ätherischen Nadelöle sind durchsichtig und normalerweise farblos. Die ätherischen Öle der Nadelbäume haben keine spezifische und dauerhafte Struktur ihre Zusammensetzung hängt oft von verschiedenen agrarökologischen Bedingungen ab. Es handelt sich um Verbindungen von Terpen-Kohlenwasserstoffen, Phenylpropanoiden und ihren Derivaten. Die Hauptinhaltsstoffe sind Pinen, Limonen, β-Phellandren, ∆3-Caren und Germacren D. In dieser Arbeit werden die ätherischen Öle von *Pinus cembra* L., *Pinus mugo* Turra, *Pinus sylvestris* L. und *Pinus nigra* J.F. Arnold unter Berücksichtigung ihrer Komponenten und ihrer biologischen Aktivität untersucht.

3 Introduction

3.1 Aromatherapy

Aromatherapy is a branch of phytotherapy and describes the use of essential oils for therapeutic purposes. After a diagnosis has been made, essential oils are dissolved in fatty plain oils and the products made from them are used in targeted therapy. Application occurs as a therapeutic measure of the sense of smell and through the skin and mucous membranes, as well as orally and in the mode of suppositories (Werner & von Braunschweig, 2006).

René-Maurice Gattefossé had coined the term in his book “Aromatherapié” in 1937. For Gattefossé, aromatherapy is based on the fragrance of essential oils as well as on natural perfume and its antimicrobial, physiological and cosmetic characteristics (Steflitsch et al., 2013).

In France, aromatherapy has been included in the medical practice since Gattefoose, and only doctors are allowed to give therapy. In England, aromatherapy is an independent healing art. In Germany, aromatherapy is not accepted the same way as in England and France, and it is not the field of preventive medicine (Beier & Wabner, 2012).

3.2 Aromatic care

Aromatic care is the professional use of essential oils in the nursing field. It is the targeted treatment of everyday ailments at home, as well as the application of oils for wellness and beauty. Essential oils are potent additions to body care and cosmetics, massage oils, baths, and fragrance lamps. They care for the skin, support the defense and self-healing powers, and serve vitalization, promotion of concentration as well as relaxation and reassurance. Aroma care is used to harmonize mood disorders such as sleep disorders, restlessness, fears, confusion, lack of appetite, depressive moods and anger. It is beneficial in case of a cold, muscle pain, skin irritations as well as small wounds (Steflitsch et al., 2013).

3.3 Essential oils

Essential oils are multicomponent systems, consisting of volatile plant constituents with mono-, sesqui- and, in some cases, diterpenes and their derivatives such as alcohols, esters, ethers/oxides, acids, phenols, lactones and phenylpropanoids (Beier & Wabner, 2012).

All plants can produce volatile compounds, but only ``Essential oil plants`` are those species that produce essential oils for commercial use. They can be produced in secretory idioblasts, cavities or ducts and secreting trichomes of a plant (glandular trichomes). Schizogenic oil ducts are typical for Pinaceae (Chlodwig & Novak, 2016).

Three different procedures are used for the preparation of essential oils: compression, water/steam distillation, and dry distillation (Schmidt et al., 2016). Water distillation is most commonly used for the extraction of essential oils (Beier & Wabner, 2012). According to the European Pharmacopoeia, only steam distillation and cold compression, in the case of citrus oils, are allowed for essential oils used for pharmaceutical and medicinal purposes (European Pharmacopoeia, 2019).

The complexity of essential oils is responsible for a variety of therapeutic and nursing properties, as an analgesic, antimicrobial, antiseptic, epithelizing, carminative, anticonvulsant, spasmolytic, hyper impressive, stimulating agent (Steflitsch et al., 2013).

Pines have been used for medical purposes since ancient times. Hippocrates used pine needle oil externally to treat ulcers. Several centuries later, there are references to the use of pine in the writings of Matthiolus and Lonicerus, describing other applications such as for liver disease, consumption, and cough. The effects of catarrhal diseases of the upper and lower respiratory tract, rheumatic complaints, and mild muscle and nerve pain are now scientifically recognized (Mayer & Niedenthal, 2018).

4 *Pinups cembra* L.

4.1 General

Pinus cembra L., also known as arolla pine or Swiss stone pine, belongs to the family of Pinaceae. It grows in the European Alps and the Carpathian Mountains at high altitudes ranging from 1200 to 2600 m. Arolla pine lives up to 1000 years and grows slowly.

Arolla pine tree can grow to be up to 25 m tall and 1.5 m wide. The leaves are 7 - 9 cm long, 1 - 1.15 mm wide, triangular in cross-section, and green. Seed cones are 5 - 8 cm long and 4 - 6 cm wide, resinous and broadly ovoid (Farjon, 2010a).

4.2 Composition of essential oils

By means of chromatographic and spectroscopic methods, such as GC-FID, GC-MS, and ^{1}H-NMR spectroscopy, 130 compounds were detected in essential pinus oils. *Pinus cembra* plant material was collected in Poland. The yields of the essential oils from twigs with needles were 0.50 %, from needles 0.46 %, from twigs without needles 0.66 %, from bark 0.64 %, from wood 0.08 %, from unripe cones 2.40 %, and from ripe cones 1.45 % (Table 1) (Lis et al., 2017).

The essential oil made from twigs with needles was dominated by α-pinene (36.3 %), limonene (22.7 %), and β-phellandrene (12.0 %), and β-pinene (4.2 %) (Figure 1), but also there were myrcene (1.0 %), camphene (1.0 %), δ-cadinene (3.8 %) as well as γ-cadinene, (E)-β-caryophyllene, α-humulene, germacrene D, α-muurolene, and epi-α-muurolol, each at concentrations around 1 – 2 %. In the needle oil there was α-pinene (48.4 %), limonene (7.5 %), and δ-cadinene (6.2 %). The oil from twigs without needles was dominated by limonene (33.6 %), α-pinene (17.5 %), β-phellandrene (17.1 %), and β-pinene (7.6 %). The bark essential oil showed limonene (36.2 %), β-phellandrene (18.8 %), and α-pinene (17.9 %) as the main constituents. The wood essential oil and cone essential oil contained α-pinene (35.2 %, 35.4 % and 39.0 %, respectively) and β-pinene (10.4 %, 21.6 %, and 18.9 %, respectively). In the wood oil thunbergol (8.4 %) was found (Lis et al., 2017).

Table 1. Chemical composition of the essential oils of *Pinus cembra* (Lis et al., 2017)

Compounds	Twigs with needls	Needls	Twigs without needls	Bark	Wood	Unripe cones	Ripe cones
Tricyclene	0.1	0.	0.1	0.1	0.1	tr	0.1
α-Pinene	36.3	48.4	17.5	17.9	35.2	35.4	39.0
Camphene	1.0	1.3	0.9	1.4	0.9	0.8	1.0
Thuja-2,4-diene	tr	0.1	0.1	0.1	tr	tr	0.4
Sabinene	tr	0.1	tr	tr	0.1	0.2	0.1
β-Pinene	4.2	1.4	7.6	4.8	10.4	21.6	18.9
Myrcene	1.0	0.7	2.1	1.7	0.7	1.9	0.5
α-Phellandrene	tr	0.1	0.1	tr	tr	0.2	0.1
3-Carene	tr	tr	tr	tr	tr	0.1	0.6
p-Cymene	tr	tr	tr	tr	tr	tr	0.1
β-Phellandrene	12,0	3.1	17.1	18.8	1.9	7.0	1.3
Limonene	22.7	7.5	33.6	36.2	6.0	21.1	3.5
(Z)-β-Ocimene	tr	0.4	tr		tr	0.1	0.1
(E)-β-Ocimene	tr	0.1	tr		tr	0.1	0.1
γ-Terpinene	0.1	tr	0.2	0.1	tr	tr	tr
Fenchone	tr		tr		tr		0.2
Terpinolene	0.1	0.2	0.2	0.2	0.2	0.1	0.1
α-Campholenal	tr		tr	tr	tr	tr	0.3
Nopinone							0.1
Camphor	tr	tr	0.1	tr	tr		0.1
trans-Pinocarveol	tr	tr	tr	tr			0.6
cis-Verbenol	tr	tr	tr	tr	tr		0.4
trans-Verbenol	tr	tr			tr		0.5
Pinocarvone					tr	tr	0.7
p-Mentha-1,5-dien-8-ol	tr		tr	tr	tr	tr	0.4
Pinocamphone	tr		tr	tr			0.1
Borneol	tr	tr	tr	tr	tr	tr	0.2
Terpinen-4-ol	tr	0.1	tr	tr	tr	0.1	0.5
Myrtenal		tr	tr	tr	tr	tr	0.3
Estragol	tr	0.1					
α-Terpineol	0.1	0.1	0.2	0.1	0.3	0.2	1.8
Myrtenol	tr	tr					0.6
Verbenone	tr	tr	tr	tr			0.2
trans-Carveol							0.2

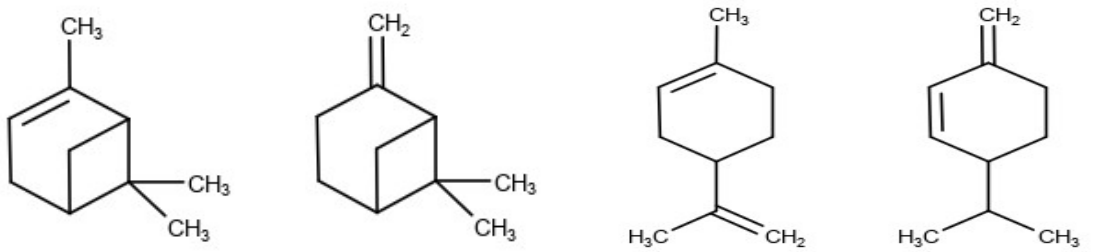
Table 1 continued

cis-Carveol							0.1
Thymol methyl ether	tr	tr	tr	tr	tr	0.2	0.2
Undecanal	0.1	0.1	0.1	0.2	tr	tr	tr
Bornyl acetate	0.2	0.4	0.2	0.4	2.6	0.6	0.7
Undecan-2-one	0.1	0.1				0.1	tr
Bicycloelemene	0.1	0.1	tr	tr	tr		
δ-Elemene	0.7	1.3	0.4	0.3	0.5		
α-Cubebene	0.1	0.1	0.1	0.1	tr		tr
α-Longipinene	tr	0.1	tr	tr	tr	tr	tr
Geranyl acetate	tr		tr	tr		0.2	tr
α-Ylangene	tr	tr	tr	tr	0.1		tr
α-Copaene	0.2	0.3	0.2	0.2	0.2		tr
Dodecan-2-one	0.1	0.1				tr	tr
β-Elemene	0.2	0.3	0.2	0.1	0.2	tr	0.1
Sibirene	tr	tr	0.1	tr	1.1		0.1
(E)-β-Caryophyllene	1.1	1.6	0.9	0.6	3.6	0.1	0.3
β-Copaene	0.2	0.2	0.1	0.1	0.1		tr
Aromadendrene	0.2	0.3	0.2	0.2	0.2		
Isogermacrene	0.1	0.1	0.1	0.1	0.1		tr
(E)-β-Farnesene	0.2	0.2	0.2	0.2	0.4	tr	tr
α-Humulene	1.0	0.4	1.9	1.6	0.9	tr	0.1
cis-Muurola-4(15),5-diene	0.2	0.3	0.2	0.1	tr		tr
γ-Muurolene	0.5	0.7	0.5	0.4	0.8	tr	0.1
Germacrene D	1.9	4.1	0.9	0.6	0.5	0.1	0.2
β-Selinene	0.1	0.1	0.1	tr	tr		
4-epi-Cubebol	0.1	0.1	tr	tr			
γ-Amorphene	0.3	0.4	0.4	0.3	0.1		
Bicyclogermacrene	0.3	0.5	0.2	0.1	0.2	tr	0.1
α-Muurolene	1.8	3.3	1.2	1.0	1.6	tr	0.2
β-Bisabolene	0.4	0.1	0.3	0.3	0.3	tr	0.1
γ-Cadinene	1.7	2.9	1.6	1.4	0.5	tr	tr
Cubebol	0.1	0.1	tr	tr			
δ-Cadinene	3.8	6.2	3.1	2.8	1.4	tr	0.1
Cadina-1,4-diene	0.1	0.2	0.1	0.1	0.3		tr
(E)-α-Bisabolene	0.4	0.6	0.5	0.3	0.8	tr	0.1
β-Caryophyllene oxide	0.1	0.1	0.1	0.1			
(E)-Nerolidol	tr	tr	tr	tr	0.1	tr	tr

Table 1 continued

trans-Dauca-4(11),7-diene	0.1		tr	0.1			
Citronellyl isovalerate	0.2	0.2	0.1	0.1	0.3	tr	tr
Germacrene D-4-ol	0.8	2.4	0.4	0.5	1.9	tr	tr
Spathulenol	0.2	0.3	tr	tr	tr	tr	tr
Caryophyllene oxide	0.3	0.2	0.4	0.5	0.3	tr	0.1
Humulene epoxide II	0.5	tr	0.9	1.2	0.2	tr	tr
6-epi-Cubenol	0.1	0.2		0.1	0.2	tr	tr
1,10-di-epi-Cubenol	0.2	0.3	0.2	0.2	0.2	tr	tr
epi-a-Muurolol	1.1	2.4	0.8	0.8	3.3	tr	0.1
α-Cadinol	0.9	2.2	0.3	0.3	1.0	tr	tr
α-Bisabolol	0.4	tr	0.3	0.5	0.5	tr	0.2
Cembrene	tr	tr	0.1	0.1	0.7	0.2	0.6
Isocembrene	tr	tr	tr	tr	0.4	0.1	0.3
Cembrene A	tr	tr	tr	tr	0.1	tr	0.1
Pimara-8(14),15-diene	tr	tr	tr	tr	0.1	tr	tr
Manool oxide	tr	tr	tr	tr	0.1	0.1	0.1
Isopimara-7,15-diene	0.1		0.1	0.1	0.5		
18-nor-Abieta-8,11,13-triene						0.1	0.9
(E)-Biformene	tr	tr	tr	tr	0.1	0.4	0.5
Abieta-8,12-diene	tr	tr	tr	tr	tr	0.1	0.2
Abietatriene	tr	tr	tr	tr	tr	tr	0.3
Thunbergol	0.1	tr	0.1	0.1	8.1	0.3	3.1
Abieta-7,13-diene	tr	tr	tr	tr	0.1	0.1	0.2
Abieta-8(14),13(15)-diene	tr	tr	tr	tr	tr	0.1	0.1
Pimaral	tr	tr	0.1	0.1	0.4	tr	0.1
Pimara-7,15-diene-3-one	tr	tr	0.1	0.1	2.1	0.5	0.3
Abieta-6,8(14)-dien-18-al	0.1	tr	0.2	0.3	2.0	5.7	10.0
Dehydroabietal	tr	tr	tr	tr	0.1	0.2	2.0
Abietal	tr	tr	tr	tr	0.4	0.3	0.4
Methyl lambertianate	0.3	0.5	0.4	0.5	0.6	0.2	0.3
Methyl dehydroabietate					0.1		tr
Dehydroabietol					tr	0.1	0.1
Abieta-8,13(15)-dien-18-al	tr	tr	tr	tr	0.5	0.8	1.2
Methyl abietate						tr	0.1
Abietol						0.1	0.1
Methyl neoabietate						tr	0.1

tr= traces



α-pinene β-pinene limonene β-phellandrene

Figure 1. Major volatile constituents of essential oils of *Pinus cembra*

Limonene, β-pinene, and camphene were predominant in levorotary forms, but α-pinene occurred in the dextrorotary way (Table 2). This enantiomeric ratio of monoterpene hydrocarbons is characteristic for essential oils of the Pinus species. The enantiomeric rate of α-pinene in all these oils depends on the species and plant origin (Lis et al., 2017).

Table 2. Enantiomeric ratio of the essential oil from twigs with needles of *Pinus cembra* (Lis et al., 2017)

Compounds	Precentage (%)
(+)-(1R,5R)-α-Pinene	92.0 %
(-)-(1S,5S)-α-Pinene	8.0 %
(+)-(R)-Camphene	20.0 %
(-)-(S)-Camphene	80.0 %
(+)-(1R,5R)-β-Pinene	4.3 %
(-)-(1R,5R)-β-Pinene	95.7 %
(+)-(R)-Limonene	1.7 %
(-)-(S)-Limonene	98.3 %

4.3 Use and scientific investigations

Swiss stone pine essential oil has low antioxidant and high antimicrobial activities. It is good against colds, sinusitis, pharyngitis, bronchitis, cough, and asthma. It strengthens the immune system, it is an expectorant, it improves breathing, and reduces the risk of infection. It also helps to repel insects (Lubinic, 1997). Additionally, one of the main indications is against burnouts, nervous depressions, and anxieties (Zimmermann, 2018).

The needles and twigs essential oils of cembran pine exhibited a weak antioxidant effect in DPPH radical scavenging tests. The EC_{50} values showed that the essential oils from needles (19.93 ± 0.75 mg/mL) and essential oils from twigs (18.66 ± 0.70 mg/mL) were less active than the positive control butylated hydroxyanisole (BHA) (3.3 ± 0.1 µg/mL). The reason for the weak antioxidant activity of *P. cembra* essential oils might be the high concentration of non-phenolic compounds, such as monoterpenes and sesquiterpenes. It is well-known that polyphenols have a tremendous potential of free radicals scavenge due the hydrogen and electron donations of their OH-groups (Lungu Apetrei et al., 2011).

The antimicrobial activities of *P. cembra* essential oils were tested by the agar diffusion assay, and the broth microdilution assay defined the minimum inhibitory concentrations (MICs) and the minimum bactericidal/fungicidal concentrations (MBCs/MFCs). The antimicrobial activities were tested in comparison to ampicillin, chloramphenicol, and nystatin (three antibiotics as references). The essential oils from twig and needles of *P. cembra* showed high activity against *Sarcina lutea* and *Staphylococcus aureus* (inhibition zones >14 mm) but no action against *Bacillus cereus*, *Escherichia coli,* and *Pseudomonas aeruginosa.* Unlike the essential oils from twigs that showed modest activity against *C. albicans* (inhibition zone of 9.66 mm), the essential oils from needles showed no antifungal activity. MIC and MBC/MFC values of cembran pine essential oils were determined in comparison to ampicillin and nystatin. The broth microdilution assay showed similar antimicrobial effects of both essential oils against *S. lutea* with MIC = 0.12 mg/mL and MBC = 0.24 mg/mL. In contrast, *S. aureus* was less responsive to these essential oils. The essential oils from twigs were more active than the oils from needles, according to MIC values (1.95 and 3.9 mg/mL, each) and MBC values (3.9 and 15.62 mg/mL, each). MIC = 7.81 mg/mL and MFC = 15.62 mg/mL values confirmed the antifungal activity of the essential oils from twigs (Tabel 3). Powerful antimicrobial effects have phenolics such as thymol, carvacrol, eugenol, and oxygenated monoterpenes such as α-terpineol, terpinen-4-ol, and linalool. The Swiss stone pine essential oil has a high content of monoterpene hydrocarbons, little content of oxygenated monoterpenes (< 1 %), and no phenolic components, because of which it probably has low antimicrobial activity. The low antimicrobial activity of monoterpenes hydrocarbons is attached to their

low hydrogen bonding capacity and water solubility. The essential oils from twigs showed more efficiency compared to that of needles. The higher percentages of limonene+β-phellandrene and β-pinene are not the only ones responsible for the antimicrobial potency of twig-oil. Synergistic, additive or antagonistic interactions between volatile constituents may also be reason for various antimicrobial activity (Lungu Apetrei et al., 2011).

Table 3. Antimicrobial activity of cembran pine essential oils determined by the broth microdilution method (Lungu Apetrei et al., 2011)

Microorganism	Essential oils from needls		Essential oils from twigs		Ampicillin		Nystatin	
	MIC mg/mL	*MBC/MFC* mg/mL	*MIC* mg/mL	*MBC/MFC* mg/mL	*MIC* mg/mL	*MBC/MFC* mg/mL	*MIC* mg/mL	*MBC/MFC* mg/mL
S. aureus	3.9	15.62	1.95	3.9	0.25	0.5	n.d.	n.d.
S. lutea	0.12	0.24	0.12	0.24	0.25	0.5	n.d.	n.d.
C. albicans	n.d.	n.d.	7.81	15.62	n.d.	n.d.	1	2

n.d.= Not determined

The insecticidal efficiency of *P. cembra* essential oils and each of the compounds were tested in various studies. In the plant, the primary function of terpenoids, as a significant compound of essential oils, is to defend it against herbivorous and pathogens. Monoterpenes interfere with the primary behavioral function of insects such as growth, development, reproduction, and also interfere with physiological and biochemical processes. α- and β-Pinene are essential constituents of the various volatile oils (Ibrahim et al., 2001). The efficiency of spraying mountain pine cones with oleoresin of Swiss stone pine cones to prevent insect attack was tested in one study, demonstrating a remarkable reduction of the overall damage to specialized cone insects. The cones sprayed with oleoresin were not damaged by insects, whereas 11 % and 31 % of the unsprayed cones were attacked by insects. α-Pinene, β-pinene, and limonene + β-phellandrene are dominant in the cone volatiles of Swiss stone pine. The cone volatiles of mountain pine showed no dominant terpenoid. The cone oleoresin of *P. cembra* significantly varied from cone volatiles by a lower level of α-pinene and higher levels of β-pinene and limonene + β-phellandrene (Dormont et al., 1997). Limonene is active against a lot of insects, mites, microorganisms and it was used as insecticide against ectoparasites of pet animals. The study showed that limonene has deterrent and insecticide characteristics. The concentration of limonene in plants has a significant

effect on the behavior and consumption of plant-feeding insects. A high concentration of limonene reduces the activity of pest insects, but the low concentration of limonene increases the activity of pest insects and it is often the signal for the insects to detect the right plant. (Ibrahim et al., 2001). Insecticidal effects of twenty-eight monoterpenes (borneol, bornyl acetate, camphene, camphor, ∆3-carene, carvone, 1,8-cineole, citronellal, β-citronellene, β-citronellol, dihydrocarvone, fenchol, fenchone, geranyl acetate, isomenthol, limonene, limonene oxide, linalool, linalyl acetate, menthol, menthone, myrcene, nerol, neryl acetate, α-pinene, β-pinene, terpinen-4-ol, α-terpineol) were tested against maize weevil. The monoterpenes were applied at contents of 10, 20 and 30 µl for liquid compounds and 10, 20 and 30 µg for solid compounds. Oxygenated monoterpenes have higher insecticidal effects than monoterpene hydrocarbons. The effect was dependent on the dosage rate and exposure time ($p < 0.01$). ∆3-Carene (monoterpene hydrocarbon) by maximum dosage of 30 µl showed 100 % of mortality after 96 hours of treatment. Oxygenated monoterpens (carvone, dihydrocarvone, menthone, and terpinen-4-ol) gave 100 % insecticidal result after 48 hours of exposure and showed the most potent insecticidal activities at all doses after 96 hours of exposure with 100 % of mortality. In comparison with other monoterpenes (1,8-cineole, fenchone, linalool, and limonene oxide) showed stronger insecticidal actions. The lethal doses (LD_{50}) values of 1.989 µl for 1,8-cineole, 2.445 µl for fenchone, 2.445 µl for linalool and 3.235 µl for limonene oxide (96 hours after treatment) on the tested insects. The toxicity of essential oils against stored pests was mainly related to their major components, which were monoterpenes. Monoterpenes from different essential oils have various toxicity ratios, and these differences derive from chemical components which are novel to them (Yildirim et al. 2013).

One study examined the effect of the essential oils of cypress and cedar from Japan. Their oil-composition is very similar to the essential oils of stone pine. The study showed that systolic blood pressure as well as activities of the brain areas decreased which were bothered with unpleasant thoughts by breathing in stone pine essential oil. A similar study examined the effect of Japanese cedar extract under stress conditions (subjects had to solve math problems) by valuing the presence of stress hormones in saliva. The increase in stress hormones was significantly lower by using the cedar essential oil than

without cedar essential oil. This reduction in stress due to the action of wood constituents was also found in studies with pinewood. Individual ingredients of tree oils such as α-pinene and limonene were also investigated in Japan. After just a few seconds a decrease in systolic blood pressure was noticed, as well as increased activity of the vagus and a reduced heart rate. This Japanese study was carried out in the period between 2008 and 2017 and it confirmed the results from earlier studies (Moser, 2019a). A sleep study was conducted, where six people slept for two nights in their own bed, then for six nights in a wooden bed, then two nights in their bed again, followed by five nights in the stone pine wooden bed, and in the end, in their own bed again. Cardiac rhythm flexibility, vagal tone and heart rate were measured. Additionally, then the participants of the study filled in a questionnaire that measured their sense of well-being while sleeping and after sleep, and brain waves were recorded using a special device. The study showed that sleeping in the stone pine bed saved an average of 3.500 heartbeats per night, which corresponds to about one hour of heartbeat. It turned out that, in addition to the decrease in heart rate, an increase in vagal tone appeared which protects the heart and prevents inflammation. Subjective sleep quality and sleeping efficiency were improved. The subjects felt better off in the morning when they slept in the wooden stone pine bed. Another study was carried out to confirm the outcome. In that study a stone pine furniture office and a classic wooden furniture office were tested. The heart rate of 40 persons was measured in these two offices, proving that it was lower when persons were working in the stone pine wooden office. Another study was done in a school. In one classroom, all the furniture and floor were made of pinewood and in the other two control classes all the furniture was made of modern materials such as plasterboard, linoleum and metal. The test was done under the hypothesis that stress and thus also heart rate particularly increase in the last weeks of school in June. The heart rate of students during the holidays and school weeks were also measured. Stress and heart rate began to increase in the control classes right at the beginning of school, and measurements showed that stress as well as heart rate was highest in June. In the classes of stone pine wood furniture, heart rate started to sink and vigil tone to raise after a few weeks of school, and in June, at the end of the school year, vigil tone was lower than in the period during the holidays. The control classes showed the lowest

values of vagal tone at the end of the school year. In contrast, stone pine classes showed unexpected relaxation and high vagal tone values (Moser, 2019b).

5 *Pinus mugo* Turra

5.1 General

Pinus mugo belongs to family Pinaceae, and it is known as dwarf mountain pine, mountain pine or mugo pine. There are two subspecies recognized: *Pinus mugo subsp. mugo* and *P. mugo subsp. rotundata*. *Pinus mugo subsp. mugo* grows in Europe at the Pyrenees, Alps, Carpathians, Dinaric Alps and central Apennines. *Pinus mugo subsp. rotundata* can be found at the Pyrenees (rare), Alps as well as in the Erzgebirge and NW Carpathians.

The hight growth for both subspecies is from 1000 to 2300 m. Mountain pine is to 5 m tall. Leaves are in fascicles of two, and they are 3 - 7 cm long and 10 - 12 mm wide, slightly curved and twisted, and also dark or light green. Seed cones are ovoid with asymmetrical or symmetrical base, 2 - 6 cm long and 2 - 4.5 cm wide (Farjon, 2010b).

5.2 Composition of essential oils

More than 130 compounds were detected by chromatographic and spectroscopic methods in essential oils of *P. mugo* from Poland. The yields of the essential oils in twigs with needles was 0.4 %, in needles 1 %, in twigs without needles 0.8 %, in wood 0.1 %, and in young shoots 1.6 % (Table 4) (Lis et al., 2019).

The essential oil from twigs with needles contained Δ3-carene (23.8 %), myrcene (22.3 %), and *α*-pinene (10.3 %), tracked by *β*-phellandrene (7.8 %), limonene (4.9 %), *β*-pinene (4.2 %), terpinolene (4.1 %), camphene (1.3 %), and sabinene (1.2 %). The main components of the needle oil were *α*-pinene (18.6 %), Δ3-carene (11.3 %), and bornyl acetate (8.3 %). In the oil twigs without needles there were Δ3-carene (28.6 %), myrcene (23.4 %), and *β*-phellandrene (8.2 %). The bark essential oil had myrcene (24.6 %) and Δ3-carene (18.5 %) and limonene, *α*-pinene, *β*-pinene, and *β*-phellandrene as the main constituents. The oil from young shoots was similar, containing myrcene (24.0 %), Δ3-carene (15.0 %) and limonene, *α*-pinene, *β*-pinene, and *β*-phellandrene as the main constituents. In the wood oil Δ3-carene (34.6 %) dominated, followed by myrcene, *α*-pinene, *α*-muurolene, and *α*-terpinyl acetate. The cone oil had essentially (*E*)-*β*-caryophyllene (24.0 %), Δ3-carene (19.2 %) and myrcene (16.5 %) (Lis et al., 2019).

Tabel 4. Chemical composition of the essential oils of *Pinus mugo* (Lis et al., 2019)

Compounds	Twigs with needls	Needls	Twigs without needls	Bark	Wood	Cones	Young shoots
Santolinatriene	tr	tr	tr	tr		tr	0.8
Tricyclene	0.3	0.7	0.1	0.1	tr	tr	0.1
α-Thujene	0.6	1.6	0.2	0.3	0.1	0.2	0.2
α -Pinene	10.6	18.6	5.5	7.1	5.7	8.9	8.0
Camphene	1.3	3.0	0.3	0.5	0.1	0.1	0.6
o-Cymene	0.1	tr	0.2	0.2	0.7	0.1	0.4
Sabinene	1.2	1.1	1.2	1.0	1.2	0.2	0.8
β- -Pinene	4.2	3.1	3.9	5.6	3.4	2.4	6.4
Myrcene	22.3	4.3	23.4	24.6	9.4	16.5	24.0
α -Phellandrene	0.6	0.6	0.6	0.6		0.1	0.5
3-Carene	23.8	11.3	28.6	18.5	34.6	19.2	15.0
α-Terpinene	0.4	0.6	tr	tr	tr	0.1	0.4
p-Cymene	0.1	0.4	0.7	0.8	0.9	0.2	0.9
β- -Phellandrene	7.8	3.6	8.2	7.7	3.6	4.5	4.2
Limonene	4.9	3.8	6.8	7.0	2.2	3.0	9.4
(E)- *β*- -Ocimene	0.1	0.4	0.1	0.1		tr	0.1
γ -Terpinene	0.4	0.5	0.2	0.4	0.1	0.2	0.2
trans-Sabinene hydrate	tr	0.1	0.1	0.1	tr		0.1
p-Cymenene				tr	tr	0.1	0.1
Isoterpinolene	0.1		tr	0.1	tr	tr	tr
Terpinolene	4.1	4.6	2.7	3.5	2.2	0.8	1.4
Linalool	tr	tr	0.1	0.2			0.1
Perillene			0.1	tr		tr	0.4
α-Fenchol		tr		0.1			0.1
cis-*p*-Menth-2-en-1-ol	0.1	0.1	0.1	0.2	0.2	tr	0.2
cis-Limonene oxide	tr		0.1	0.1	0.2	tr	0.2
trans-*p*-Menth-2-en-1-ol	0.1	0.1	0.1	tr			
trans-Pinocarveol	tr		tr	0.1	0.3	tr	0.4
p-Mentha-1,5-dien-8-ol	0.1	0.1	tr	0.5	0.4	tr	0.3
cis-Verbenol				0.1			0.4
Borneol	0.1	0.2	0.1	0.4	0.1	0.1	0.4
Cryptone			0.1		0.3		
Terpinen-4-ol	0.5	0.9	0.7	1.3	3.0	0.3	1.2
p-Cymen-8-ol	0.2	0.1	tr	0.5	0.1	tr	0.6

Table 4 continued

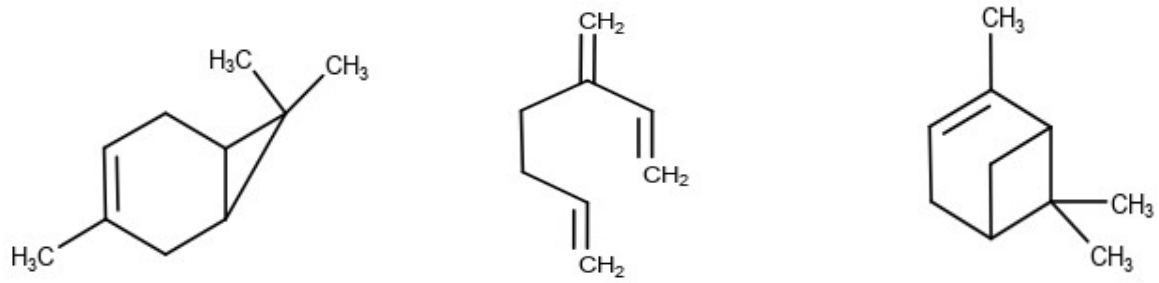
Myrtenal					0.3	tr	0.2
α-Terpineol	0.2	0.4	0.5	1.6	0.8	0.1	0.3
Myrtenol	tr	tr	0.1	0.1	0.3	tr	0.2
Verbenone	tr	tr	0.1	0.1			0.4
trans-Piperitol	tr	0.1	tr	0.1	0.1		0.1
trans-Carveol				tr			0.3
Fenchyl acetate	tr	tr	tr	tr			0.1
Citronellol	tr		0.1	tr			0.3
Thymol methyl ether	0.3	0.1	0.6	0.6	0.4	0.3	0.9
Piperitone			tr	0.1	0.3		tr
Linalyl acetate	tr	tr	0.1	tr	0.1		0.2
Bornyl acetate	1.5	8.3	1.4	2.1	0.6	tr	2.4
Undecan-2-one	0.1	0.4	0.1	0.1			0.3
cis-Pinocarvyl acetate	tr	tr	tr	0.1	0.1	tr	tr
δ-Terpinyl acetate	0.2	0.1	0.3	0.3	2.0	0.1	0.2
α-Terpinyl acetate	2.4	2.8	2.1	2.2	4.3	0.4	1.9
Citronellyl acetate	0.2	tr	0.1	tr	0.1		0.1
α -Cubebene	tr	tr	tr	0.1	0.2	0.4	
α -Copaene	0.1	0.1	0.1	0.1	0.5	0.4	0.1
α -Elemene	tr	0.1	tr	tr			
β-Bourbonene	tr	0.1	tr	tr			0.1
β-Elemene	0.5	1.5	0.3	0.5	0.2	0.1	tr
(E)- *β*- -Caryophyllene	0.8	2.8	0.6	0.6	0.2	24.0	0.3
β -Copaene	0.1	0.2	0.2	0.3	0.6		tr
Isogermacrene	tr	0.1	tr	0.1			
(E)- *β* -Farnesene						0.2	0.1
α-Humulene	0.2	0.6	0.1	0.2	0.1	3.8	0.1
cis-Muurola-4(15),5-diene	tr	0.1	tr	tr			
2-*epi*-(*E*)-*β*-Caryophyllene	tr	0.2	tr	tr		1.1	tr
γ -Muurolene	0.2	0.4	0.2	0.3	0.5	0.1	0.1
Germacrene D	1.7	4.6	1.0	1.6	0.9	0.2	0.1
β -Selinene	0.1	0.2	0.2	0.2	0.1	0.1	tr
epi-Cubebol	tr	tr	tr	tr		0.1	
γ -Amorphene	tr	0.2	tr	0.1			
Bicyclogermacrene	0.2	0.7	tr	0.2			
α -Muurolene	0.7	1.1	1.1	1.2	4.5	0.3	0.3
δ-Amorphene	tr	0.1	tr	tr			

Table 4 continued							
Bornyl isovalerate	0.1	0.4	tr	0.1	tr		0.1
γ -Cadinene	0.1	0.5	0.1	0.2	0.1	0.4	tr
δ Cadinene	0.4	2.1	0.2	0.4	0.2	0.2	tr
Cadina-1,4-diene	tr	0.1	tr	tr		0.1	
α-Cadinene	tr	0.1	tr	tr		tr	
β -Caryophyllene oxide	tr	0.1	tr	tr	0.2	tr	
(E)-Nerolidol	tr	0.1	tr	tr			tr
Citronellyl isovalerate	0.1	0.1	0.2	0.2	tr		0.2
Germacrene D-4-ol	tr	tr	tr	tr	tr		
Spathulenol	0.3	1.6	0.2	0.2	0.1		tr
Caryophyllene oxide	0.1	0.2	0.2	0.1	0.2	0.6	0.1
Salvial-4(14)-en-1-one	tr	0.1	tr	tr	0.1		
Geranyl 2-methylbutyrate	0.1	0.1	0.1	0.2	0.3		0.1
Viridiflorol	0.1	0.1	0.1	0.1	0.9		tr
β-Oplopenone	tr	0.1					
Longiborneol	0.1	0.1	0.1	0.1			
Humulene epoxide II	tr					0.1	tr
6-*epi*-Cubenol	tr	0.1					
1,10-di-*epi*-Cubenol	tr	0.2	tr	tr		0.1	
Muurola-4,10(14)-dien-1-ol	tr		tr		1.1		
epi-*α*-Muurolol	0.3	2.6	0.1	0.2	tr	0.1	tr
α-Muurolol	0.1	tr	0.1	0.2	0.6	tr	tr
2-*epi*-Thujopsa-3-one			tr		0.5		
α-Cadinol	0.6	3.2				tr	0.1
Eudesm-11-en-4*α*-ol	tr	tr	0.5	0.6			
14-Hyrdoxy-*β*-caryophyllene	tr	0.1	tr	tr			
Eudesm-7(11)-en-4-ol	tr		0.1	0.1	0.2		
Germacra-4(15),5,10(14)-trien-1*α*-ol	tr	0.1	tr		0.7		
trien-1*α*-ol							
Oplopanone	tr	0.2	tr	tr	0.1		
Isopimara-9(11),15-diene							0.2
Cembrene	tr	tr	0.1	0.1	0.1	tr	0.2
m-Camphorene	0.1	tr	0.2	0.2		tr	0.3
Pimara-8(14),15-diene	tr	tr	tr	0.1	0.3	tr	0.1
p-Camphorene	tr	tr	0.1	0.1		tr	0.2
Manool oxide	tr	0.3	tr	tr	tr	tr	0.2

Table 4 continued							
Isopimara-7,15-diene	0.1	tr	0.1	0.1	0.2	tr	0.3
18-nor-Abieta-8,11,13-triene	tr	tr	0.1	tr	0.2	0.1	1.2
(*E*)-Biformene	tr	tr	tr	tr	0.1	0.5	0.1
Abieta-8,12-diene	tr	tr	tr	tr	0.1	0.2	0.1
Abietatriene	tr	tr	tr	tr	0.1	0.1	0.1
Thunbergol	tr	tr	tr	tr	0.3	tr	0.1
Abieta-7,13-diene	tr	0.2	tr	tr	0.1	0.6	0.1
Linoleic acid	tr	tr	0.3	tr	tr	0.1	0.1
Abieta-8(14),13(15)-diene	tr	tr	tr	tr	tr	0.2	0.1
Pimaral	0.1	tr	0.1	0.1	0.8	0.1	0.2
2-Oxomanoyl oxide	tr	0.4					
Abieta-6(8),14-dien-18-al	0.3	0.2	0.5	0.5	1.0	4.8	1.8
Dehydroabietal	tr	tr	0.3	0.1	1.9	0.7	0.9
Sandaracopimarinol	tr		tr		0.7		0.6
Methyl isopimarate	tr	tr	tr	0.1	0.1	tr	tr
Abietal	tr	tr	tr	0.1	0.2		0.2
Abieta-8,13-dien-18-ol						0.5	0.1
Methyl dehydroabietate	tr	tr	0.1	0.1	0.3	tr	0.1
Dehydroabietol	tr		tr		0.3		0.1
Abieta-8,13(15)-dien-18-al	tr	tr	tr	0.1	tr	0.7	0.3
Abietol						0.1	

tr= traces

The principal constituents of the oils were Δ3-carene, myrcene, and *α*-pinene (Figure 2), pursued by *β*-phellandrene, limonene, *β*-pinene, terpinolene, camphene, and sabinene (Lis et al., 2019).



Δ3-carene myrcene α-pinene

Figure 2. Major volatile constituents of essential oils of *Pinus mugo*

Limonene, *β*-pinene, and camphene dominated in the levorotary more than in the dextrorotary forms. *α*-pinene comes in both forms, and ∆3-carene occurs only in the dextrorotary form (Table 5) (Lis et al., 2019).

Table 5. Enantiomeric ratio of the essential oil from twigs with needles of *Pinus mugo* (Lis et al., 2019)

Compounds	**Precentage (%)**
(+)-(1R,5R)-α-Pinene	60.4
(-)-(1S,5S)-α-Pinene	39.6
(+)-(R)-Camphene	11.2
(-)-(S)-Camphene	88.8
(+)-(1R,5R)-β-Pinene	5.7
(-)-(1R,5R)-β-Pinene	94.2
(+)-(*S*)-3-Carene	100.0
(+)-(R)-Limonene	28.0
(-)-(S)-Limonene	73.0

5.3 Use and scientific investigations

The essential oil of *P. mugo* is often topicaly used in different medicinal preparations. It is a supportive therapy in rheumatic diseases because of the stimulation of blood circulation, which is principally caused by Δ3-carene. In combination with α-tocopherol and β-carotene, terpinolene, which are contained in the essential oil of *P. mugo*, it efficiently prevents oxidation of LDL (Karapandzova et al., 2018). Essential properties are antiseptic, anti-inflammatory, secretolytic, slightly hyperemising (especially on the respiratory tract) and immunomodulatory activity. The main indications are rhinitis, bronchitis cystitis, immune deficiency, rheumatitis, gout and sore muscles (Zimmermann, 2018).

The essential oils of the twigs of three Pinus species (*P. mugo*, *P. peuce* and *P. heldreichii*) and the cones of *P. mugo* exhibited notable anti-inflammatory activity in lipopolysaccharide (LPS) stimulated macrophages. Essential oils from needles of these three Pinus species reduced the secretion of the pro-inflammatory cytokine IL-6 (interleukin 6) by at least 35 % at a concentration of 0.001 %. The oils derived from twigs of these three Pinus species and the cones of *P. mugo* lowered the secretion of IL-6 by

at least 60 % at a concentration of 0.01 % (Figure 3). The needles essential oils of *P. peuce* and *P. heldreichii* showed the lowest IC_{50} of 0.0008 %, whereas the oils of *P. mugo* cones had the highest IC_{50} of 0.02 % (Table 6). According to the previous studies, it can be assumed that α-pinene, as a major compound in all Pinus species may influence the secretion of cytokines and may play a significant role in the anti-inflammatory activity (Basholli-Salihu et al., 2017).

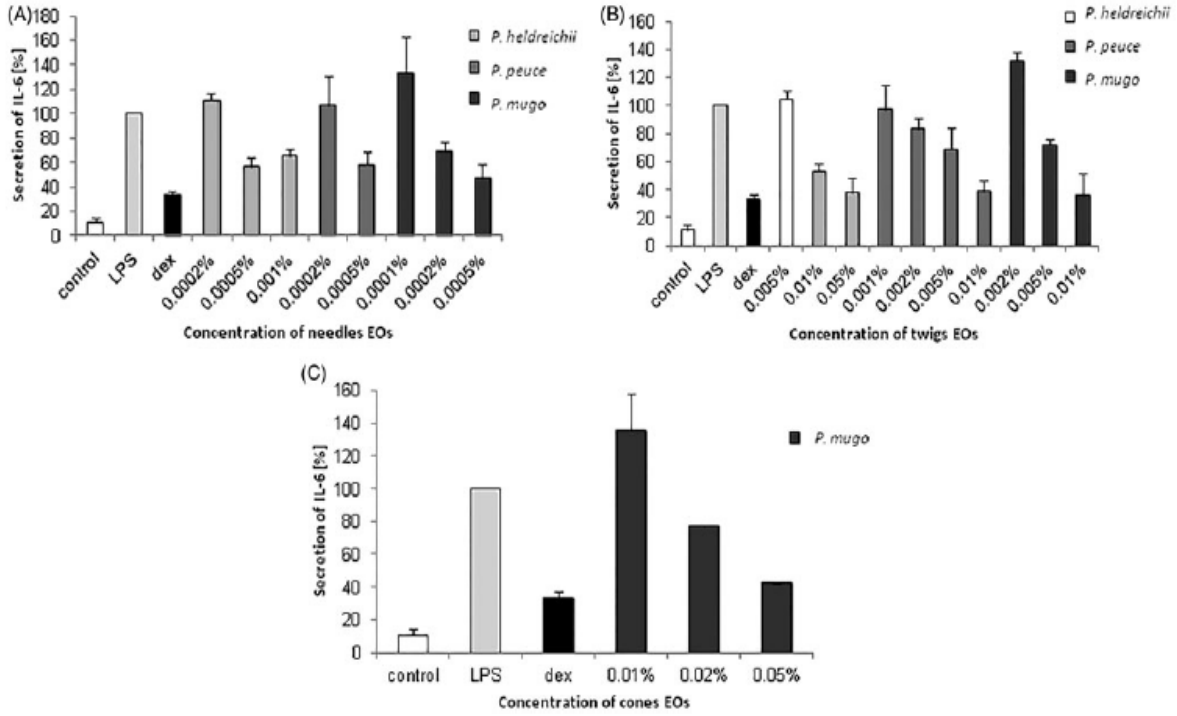


Figure 3. Concentration-depended reduction of IL-6 secretion of LPS-stimulated macrophages, as determined by ELISA, after treatment with (A) the needle and (B) twig oils of *P. heldreichii*, *P. peuce* and *P. mugo*, (C) the cone oils of *P. mugo* and 5 IM dexamethasone (dex) (Basholli-Salihua et al., 2017)

Table 6. IC_{50} values [%] of *P. heldreichii*, *P. peuce* and *P. mugo* for IL-6 reduction (Basholli-Salihua et al., 2017)

Substances	IC_{50} [%]
P. heldreichii twigs	$6*10^{-3}$
P. heldreichii needles	$2*10^{-4}$
P. peuce twigs	$8*10^{-4}$
P. peuce needles	$> 5*10^{-4}$
P. mugo twigs	$4*10^{-3}$
P. mugo needles	$2*10^{-4}$
P. mugo cones	$2*10^{-2}$

All essential oils exhibited a notable cytotoxic effect on cancer cell lines HeLa, CaCo-2 and MCF-7. The essential oils of the needles and twigs from *P. peuce* and *P. mugo*, and needles of *P. heldreichii* showed the most significant cytotoxic activity on HeLa and CaCo-2 cell lines. *P. peuce* (twigs and needles) and *P. mugo* needles showed the most substantial activity on MCF-7 cell lines. Essential oils of the needles and twigs from *P. peuce* and *P. mugo* and essential oils of *P. heldreichii* needles reduced cell viability of HeLa up to 90 % at a concentration of 0.1%, but the essential oils of the needles of *P. heldreichii*, at the same concertation, lead to a reduction of only by 60 %. The lowest IC_{50} value by HeLa was noticed by the needles of *P. peuce* with IC_{50} of 0.007, but the highest value had *P. mugo* needle essential oils with IC_{50} of 0.3. The essential oils from *P. peuce* needles and *P. mugo* needles and twigs decreased the cell viability of CaCo-2 by 80–90 %. These were followed by the oils of *P. heldreichii* twigs and oils of *P. mugo* cones which decreased the cell viability of CaCo-2 nearly 50 – 65 %. The lowest IC_{50} value in CaCo-2 cell lines was determined for essential oils of *P. mugo* twigs with IC_{50} of 0.06, but the highest IC_{50} value had the essential olis of *P. heldreichii* twigs and *P. mugo* cones with IC_{50} of > 0.1. The essential oils of *P. peuce* twigs, *P. peuce* needles, and *P. mugo* needles reduced cell viability of MCF-7 cell lines by at least 85 %. The lowest IC_{50} value in MCF-7 cell lines were found for the essential oils of needles and twigs *of P. peuce* with IC_{50} of 0.06, but essential oils of *P. mugo* needles showed the highest IC_{50} of 0.3 (Table 7). Plant extracts that show cytotoxicity with an IC_{50} of < 30 µg/mL can be considered as potential agents for the development of anticancer drugs, according to the American National Center Institute. Therefore, the essential oils of *P. peuce* needles, *P. peuce* twigs and *P. heldreichii* needles with IC_{50} lower than 0.03 µg/mL can be considered as promising agents for anticancer drug development for HeLa cell lines, whereas the essential oils from *P. mugo*, *P. peuce*, and *P. heldreichii* needles can be considered as promising agents for anticancer drug development for CaCo-2 cell lines (Basholli-Salihu et al., 2017).

Table 7. IC_{50} values [%] of *P. heldreichii*, *P. peuce* and *P. mugo* for cytotoxicity (Basholli-Salihua et al., 2017)

Substances	IC_{50} [%] To HeLa	IC_{50} [%] To CaCo-2	IC_{50} [%] To MCF-7
P. heldreichii twigs	$6*10^{-2}$	$> 1*10^{-1}$	$> 1*10^{-1}$
P. heldreichii needles	$2*10^{-2}$	$2*10^{-2}$	$> 1*10^{-1}$
P. peuce twigs	$2*10^{-2}$	$4*10^{-2}$	$6*10^{-2}$
P. peuce needles	$7*10^{-3}$	$2*10^{-2}$	$6*10^{-2}$
P. mugo twigs	$6*10^{-2}$	$6*10^{-2}$	$> 1*10^{-1}$
P. mugo needles	$3*10^{-1}$	$2*10^{-2}$	$3*10^{-1}$
P. mugo cones	$> 1*10^{-1}$	$> 1*10^{-1}$	$> 1*10^{-1}$

One study proved that pine needle oil induced G2/M cell cycle arrest in HepG2 cells by activating the ATM pathway and showed an anticancer effect. G2/M arrest is an efficient tactic for controlling cancer cells-proliferation. G2/M cell cycle arrest is one of two cell cycle checkpoints that occur when DNA is damaged and allows DNA to repair. Ataxia-telangiectasia mutated (ATM) as serine/threonine protein kinase phosphorylates various proteins that activate the DNA damage checkpoint and DNA repair. Some of these proteins are p53, checkpoint kinase 2 (CHK2), breast cancer 1, nibrin 1, and H2A histone family, member X (H2AX). They are tumor suppressors. Accordingly, dysregulation of ATM can cause DNA damage, which leads to cancer. The pine needle oil-treated HepG2 cells showed increased level of phosphorylated proteins such as, p-ATM, γ-H2A histone family, member X, p-p53, p-checkpoint kinase 2 and p-cell division cycle 25C. Therefore, the results revealed that pine needle oil aided G2/M arrest in HepG2 cells by the ATM pathway. The confocal laser scanning microscopy data confirmed that treatment with pine needle oil increased p-ATM in the nucleus of HepG2 cells, showing that DNA damage induced by phosphorylation in the core of the activation of ATM. Based on the data it can be concluded that by activating the ATM signaling pathways pine needle oil causes G2/M cell cycle arrest, then inhibits proliferation, and coordinates proliferation and apoptosis in HepG2 cells. The number of HepG2 cells at the G2/M phase was distinctly higher when the cells were treated with pine needle oil

(Table 8). The results indicated that pine needle oil induced in HepG2 cells, caused cell cycle arrest at the G2/M checkpoint, and inhibited cancer cell growth by mitotic progression (Qiu et al., 2017).

Table 8. Cell cycle analysis of HepG2 cells treated with pine needle oil (Qiu et al.,2017)

Cell cycle phase	Percentage of cells in each cell cycle phase in Dimethylsulfoxide	Percentage of cells in each cell cycle phase in Pine neele oil (0,16 mg/ml)	Percentage of cells in each cell cycle phase in Pine needle oil (5,12 mg/ml)
G1	61.5 ± 5.2	47.4 ± 3.9[a]	43.2 ± 1.3[b,c]
S	21.5 ± 4.4	17.2 ± 2.4[a]	11.3 ± 1.6[b,c]
G2/M	17.0 ± 1.1	35.4 ± 3.4[a]	45.5 ± 2.1[b,c]

Data were produced from three independent experiments and are presented as the mean ± standard error of the mean. [a]P<0.05 low dose pine needle oil group vs. DMSO group; [b]P<0.05 high dose pine needle oil group vs. DMSO group; [c]P<0.05 high dose pine needle oil group vs. low dose pine needle oil group

The antioxidative activity of *P. mugo* essential oils and its major components is not extremely strong in hydrophilic surrounding, like in lipophilic surrounding such as the Fenton system, xanthine oxidase induced superoxide radical formation, or in the HOCl driven fragmentation of 1-aminocyclopropane-1-carboxylic acid (ACC). Some major components of *P. mugo* essential oil, such as ∆3-carene, camphene, *R*-pinene, (+)-limonene and terpinolene were also tested. It was shown that the antioxidative activity of *P. mugo* essential oil and its major components was not extremely strong in the hydrophilic surrounding. IC_{50} values were not reached by reasonable concentrations and only IC_{25} values could be given. *Pinus mugo* essential oil and its major components reacted only slowly with the other tested reactive oxygen species (ROS) and also exhibited no inhibition of any of the used enzymes (Table 9). Anyway, in tests conducted in more lipophilic environments (e.g., the ACC-cleavage by activated neutrophils in whole blood as well in the copper induced oxidation of LDL) *P. mugo* essential oil demonstrated good antioxidative activity. The essential oil as well as the monoterpenes *R*-pinene, (+)-limonene, and ∆3-carene inhibited ethylene generation from ACC in the whole blood as an implication for myeloperoxidase activity after degranulation. The effect of *P. mugo* essential oil or its components on the signal cascade between the initial zymosan recognition and degranulation can be the reason for inhibition of ethylene formation from ACC. The results also proved that incubation of blood plasma with *P.*

mugo essential oil or main components lead to an accumulation of terpenoids in LDL and notable protection of LDL against copper-induced oxidation. Copper-catalyzed diene conjugation has been used as a model system for testing LDL oxidation. The human blood plasma was incubated with various concentrations of *P. mugo* essential oils (0.01 % up to 0.25 % v/v). 0.01 % *Pinus mugo* essential oils gave a prolongation of the lag time of 4 min, but 0.25 % *P. mugo* essential oils gave prolongation of the lag time of 48 min (Figure 4). LDL was also incubated with the characteristic monoterpenes, such as α-pinene, Δ3-carene, camphene, and terpinolene. The first three compounds showed weak effects, but terpinolene demonstrated a clear and strong delay of diene conjugation. 0.25 % terpinolene gave the prolongation of the lag time of 774 min. The terpinolene concentration was in a linear correlation with the prolongation of the lag time. Furthermore, terpinolene was compared with its isomer, γ-terpinene, which showed a much stronger effect as terpinolene. 0.025 % terpinolene or γ-terpinene furnished a lag time prolongation of 112 and 177 min, each (Figure 5). Incubation of human blood plasma with 0.5 ‰ terpinolene or γ-terpinene incorporated 90 or 100 molecules of terpene per LDL particle. Terpinolene or γ-terpinene again confirmed the outstanding properties of those terpenoids in a lipophilic environment. Bis-allylic carbons are potential electron donors for free radicals such as alkoxyl- and hydroperoxyl, therefore terminating chain reactions. It is assumed that γ-terpinene retards peroxidation of linoleic acid via chain-carrying HOO radicals, which react quickly with linolylperoxyl radicals. The bis-allylic carbons are crucial for this reaction because they allow the aromatization of γ-terpinene to p-cymene. The protection of lipids in the skin and other organs may proceed via a similar mechanism, so furnishing proper protection against various exogenous attacks (Grassmann et al., 2003).

Table 9. IC_{25} Values of *Pinus mugo* essential oils and its components in different test systems for antioxidative capacity (Grassmann et al., 2003)

Test system	***Pinus mugo* essential oils**	**Δ3-carene**	**α-pinene**	**(+)-limonene**
xanthine/xanthinoxidase KMB	0.164 ± 0.03	0.098 ± 0.023	no reaction	no reaction
xanthine/xanthinoxidase hydroxylamine	0.06 ± 0.009	0.337 ± 0.016	no reaction	no reaction
NADH/diaphorase-KMB	0.397 ± 0.146	0.071 ± 0.03	no reaction	no reaction
Fenton-KMB	0.180 ± 0.025	0.029 ± 0.026	no reaction	no reaction
SIN-1-KMB	0.208 ± 0.023	0.259 ± 0.090	no reaction	no reaction

HOCl-ACC	no reaction	no reaction	no reaction	no reaction

Figure 4. Influence of *Pinus mugo* essential oils (PMEO) on cooper-induced formation of conjugated dienes LDL (Grassmann et al., 2003)

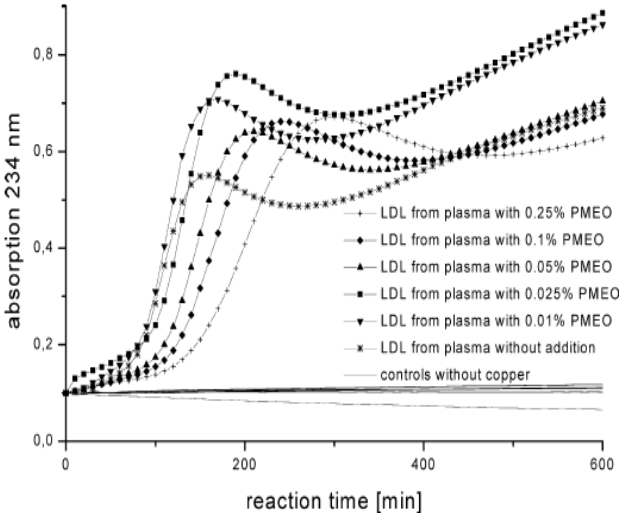
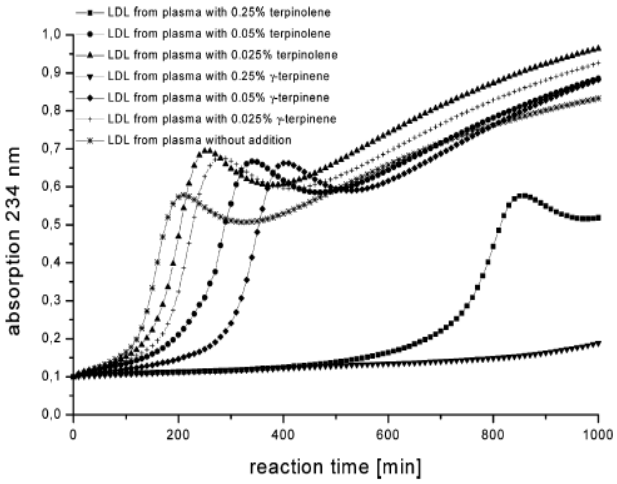


Figure 5. Influence of terpinolene and γ-terpinene on induced formation of conjugated dienes in LDL (Grassmann et al., 2003)



Major volatile constituents of essential oils of *P. mugo* are ∆3-carene, myrcene, and *α*-pinene. Monoterpenes Δ3-carene and α-pinene are very good for relaxing, sleep disorders or anxiety. Two separate studies demonstrated that Δ3-carene and α-pinene from pine tree had a sleep-enhancing effect by targeting $GABA_A$-benzodiazepine receptors ($GABA_A$-BzR). The sleep-enhancing effect of ∆3-carene was tested by a pentobarbital-induced sleep test in imprinting control region (ICR) mice. ∆3-Carene was orally applied. Zolpidem (10 mg/kg, p.o.) was used as control which is a famous hypnotic drug and positive modulator of $GABA_A$-benzodiazepine receptors. Zolpidem significantly decreased sleep latency (2.53 ± 0.08 min) and increased sleep duration (132.1 ± 15.48 min). The study showed that ∆3-carene (100 mg/kg, p.o.) had a similar dose-dependent effect in sleep latency and duration as Zolpidem (sleep latency: 2.62 ± 0.05 min; sleep duration: 122.2 ± 8.74 min) (Figure 6). Molecular mechanism of ∆3-carene was investigated by treatment with Flumazenil (antagonist of $GABA_A$-benzodiazepine receptor), 15 min before administration of ∆3-carene, and then sleep behavior was measured. The hypnotic effect of Zolpidem was antagonized with Flumazenil (1 mg/kg) without affecting sleep latency and duration. Flumazenil inhibited the hypnotic effect of ∆3-carene, insinuating that ∆3-carene can be a positive modulator of the $GABA_A$-BZD

receptor (Figure 7). It was also proven that Δ3-carene binds at benzodiazepine site of α1 and γ2 subunits of $GABA_A$ receptors. The results showed that Δ3-carene had a sleep-enhancing effect by acting as a positive modulator for $GABA_A$-benzodiazepine receptor, as α-pinene (Woo et al., 2019).

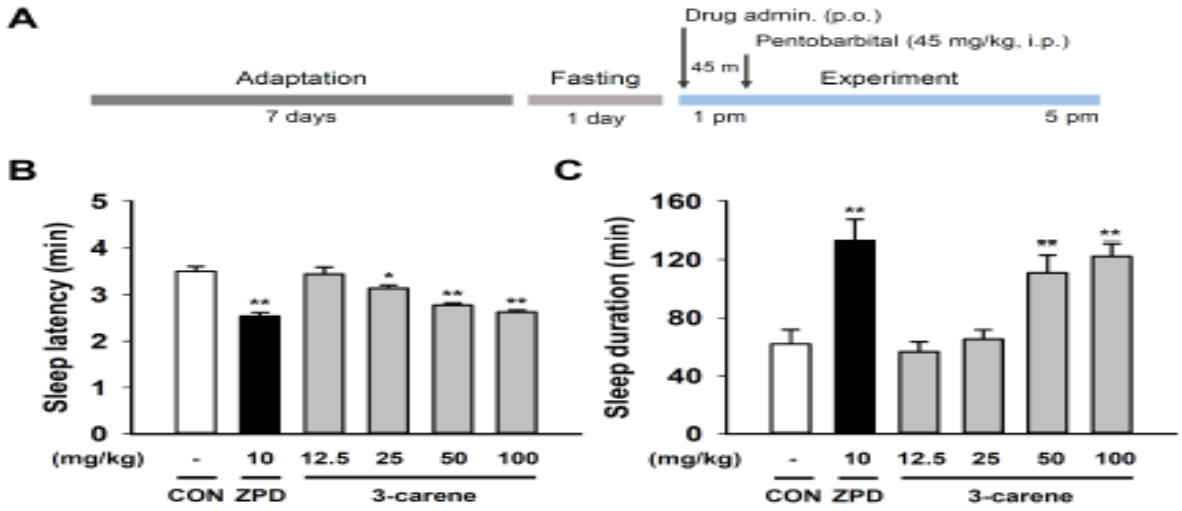


Figure 6. (A) Experimental procedure for sleep test. Effects of Δ3-carene on sleep latency (B) and sleep duration (C) in mice induced by hypnotic dose (45 mg/ kg, i.p.) of pentobarbital. Each column represents mean±SEM (n=10). *p<0.05, **p<0.01, significant as compared to the control group (One-way ANOVA, Dunnett's test). Con, control group; ZPD, zolpidem group (Woo et al., 2019)

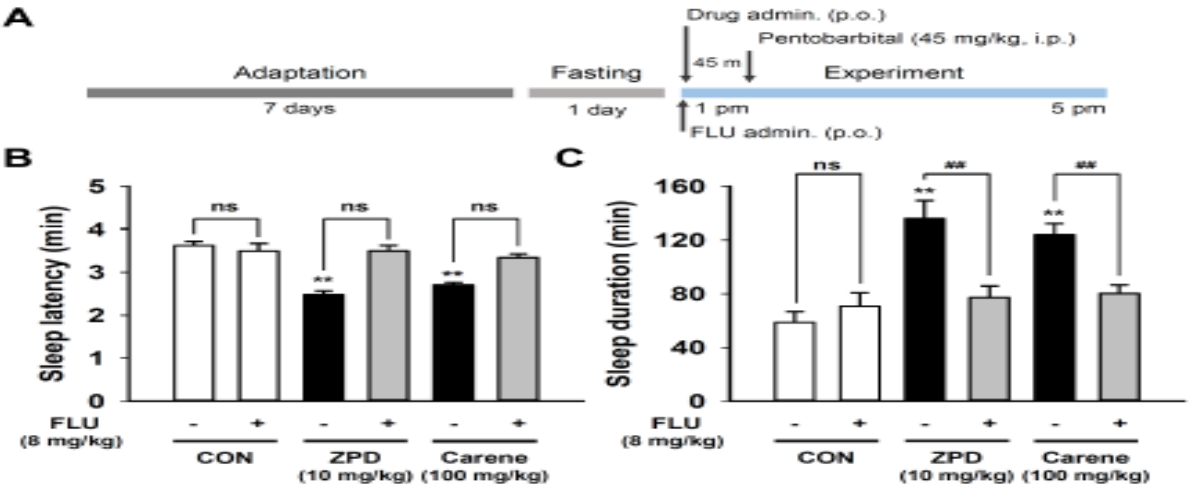


Figure 7. (A) Experimental procedure for sleep test. Effects of FLU on the changes in sleep latency (B) and sleep duration (C) in mice treated with ZPD and Δ3-carene. FLU (8 mg/kg, i.p.) was administered 10 min before oral administration. Each column represents mean±SEM (n=10). **p<0.01, significant as compared to the control group (Oneway ANOVA, Dunnett's test). ##p<0.01, significant between FLU treatment and no FLU treatment (unpaired Student's ttest). CON, control group; FLU, flumazenil; NS, not significant; ZPD, zolpidem group (Woo et al., 2019)

6 *Pinus sylvestris* L.

6.1 General

Pinus sylvestris L. is known as Scots pine. It is widespread to 2600 m in Eurasia from North Spain and Scotland to Russia (West→East), as well from Lapland to Turkey (North→South). Scots pine has 50 subspecies that have been named and described.

Scots pine is up to 35 - 40 m tall and up to 1.5 m wide. Leaves are in fascicles of two, 4 - 7 cm long and 1 - 2 mm wide, straight, rigid, and slightly twisted. Seed cones are ovoid-conical, near - symmetrical, 3 - 7 cm long and 2 - 3 cm wide (Farjon, 2010c).

6.2 Composition of essential oils

Essential oils of *P. sylvestris* was identified by GC and GC–MS analyses (Table 10). Fresh needles were collected from wild-growing populations from the central Balkan and from Northern Lithuania.

The main components of the needle oil from central Balkan were *α*-pinene (41.9 %), *β*-pinene (3.2 %) and ∆3-carene (3.6 %) (Mitić et al., 2018) and from northern Lithuania *α*-pinene (7 %), and ∆3-carene (11.4 %). Analysis of volatile compounds of Scots pine seeds showed that major constituents were ∆-3-carene, α-pinene, and β-pinene (Figure 8). Differences in concentrations of secondary compounds in pine needles slightly varied depending on the territory from which the samples were collected, depending on climatic and soil varieties (Judzentiene et al., 2008).

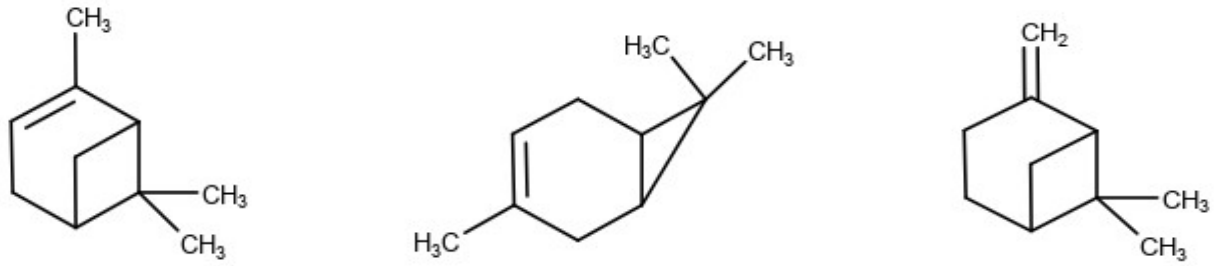
Tabel 10. Chemical composition of the essential oils of *Pinus sylvestris* from Balkan and Lithuania

Compounds	**Needls from central Balkan** (Mitić et al., 2018)	**Needles from Northern Lithuania** (Judzentiene et al., 2008)
(Z)-Pent-2-en-1-ol	tr	
n-Hexanal	tr	
(Z)-Hex-2-en-1-al	0.1	
Santene	tr	
Tricyclene	1.2	0.1
α-Thujene	0.1	
α-Pinene	41.9	7.0
Camphene	4.7	1.8
Thuja-2,4(10)-diene	tr	
Sabinene	0.1	0.7
β-Pinene	3.2	
β-Myrcene	1.9	1.0
α-Phellandrene	0.1	
δ-3-Carene	3.6	11.4
α-Terpinene	0.1	
p-Cymene	0.1	0.3
Limonene + β-Phellandrene	2.0	0.7
(Z)-β-Ocimene	0.1	tr
(E)-β-Ocimene	2.0	0.8
γ-Terpinene	0.2	0.4
Terpinolene	0.6	2.2
n-Nonanal	tr	
endo-Fenchol	tr	
α-Campholenal	tr	
trans-Pinocarveol	0.1	
trans-Verbenol	tr	
Camphene hydrate	0.1	
Pinocarvone	tr	
Undecane		0.6
Borneol	tr	0.1
p-Mentha-1,5-dien-8-ol	tr	0.2
Terpinen-4-ol	0.1	0.7
m-Cymen-8-ol		tr
p-Cymen-8-ol	tr	0.1

Table 10 continued		
α-Terpineol	0.2	0.5
Myrtenol	0.1	
Verbenone	tr	
trans-Carveol	tr	
2-Decanone		0.2
Dodecane		0.2
Methyl Thymol	tr	0.3
Decanol		0.1
Carvone	tr	
Bornyl acetate	1.9	1.2
2-Undecanone	0.1	0.4
Bicycloelemene	tr	
(E,E)-2,4-Decadienal		1.2
δ-Elemene		0.6
α-Terpinyl acetate	0.3	1.1
α-Cubebene	tr	
α-Ylangene	tr	
α-Copaene	0.2	0.1
β-Bourbonene	0.1	tr
β-Elemene	0.6	0.9
β-Ylangene	6.0	
Tetradecanene		0.4
β -Caryophyllene	0.1	5.8
β-Copaene	0.2	tr
β-Gurjunene		0.1
Aromadendrene	0.1	0.1
cis-Muurola-3,5-diene	0.1	
trans-Muurola-3,5-diene	1.2	tr
α-Humulene	0.2	1.5
9-epi-(E)-Caryophyllene	0.2	
trans-Cadina-1(6),4-diene	0.6	
(E)-β-Farnesene		tr
cis-muurola-4(14),5-diene		0.2
γ-Muurolene	3.0	1.5
Germacrene D		1.8
2-Phenylethyl 2-methylbutanoate	0.6	

Table 10 continued		
β-Selinene	0.2	0.5
Trans-muurola-4(14),5-diene		0.1
γ-Amorphene	2.9	
Bicyclogermacrene	1.0	1.2
α-Muurolene	0.3	1.2
δ-Amorphene	2.5	
γ-Cadinene	6.0	2.2
δ-Cadinene	0.1	3.0
Zonarene	0.2	
Trans-cadina-1(2),4-diene		0.2
α-Cadinene	0.3	0.2
α-Calacorene	tr	0.1
β-Calacorene	tr	tr
(Z)-3-hexenyl benzoate		0.2
Germacrene D-4-ol	0.4	0.5
Spathulenol	0.4	0.7
Caryophyllene oxide	0.2	0.6
Viridiflorol	tr	
Caryophyllene oxide		0.6
Gleenol		0.2
β-Oplopenone	tr	0.1
1,10-di-epi-Cubenol	0.1	0.3
1-epi-Cubenol	0.2	0.6
epi-α-Murrolol	2.0	
α-Murrolol	0.3	
α-Cadinol	2.0	8.5
epi-α-cadinol+ epi-α-muurolol+α-muurolol		9.1
Cembrene	0.2	
Manoyl oxide	tr	0.4
13-epi-Manool oxide	2.4	
Abietadiene		0.2
Unknown 1		12.5
Abieta-8(14),13(15)-diene		0.2

tr= traces



α-pinene β-pinene Δ3-carene

Figure 8. Major volatile constituents of essential oils of *Pinus sylvestris*

α-Pinene and *β*-pinene dominated in the levorotary more than in the dextrorotary forms and Δ3-carene occured in the dextrorotary form. Only *α*-pinene revealed variations in the enantiomeric ratio percentages (Tabel 11). Many factors such as plant tissue sampled, sample scale, geographic position, methodology, chemotypes, abiotic, and biotic stress factors can provide diversity in the quantitative enantiomeric configuration (Rodrigues et al., 2017).

Table 11. Enantiomeric ratio of the essential oil from twigs with needles of *Pinus sylvestris* (Rodrigues et al., 2017)

Compounds	**Precentage (%)**
(+)-α-Pinene	27.2 ± 5.3
(-)-α-Pinene	72.8 ± 5.3
(+)-β-Pinene	0.9 ± 0.7
(-)-β-Pinene	99.1 ± 0.7
(+)-Δ3-Carene	100.0 ± 0.0
(-)-Δ3-Carene	tr
(+)-Limonene	n.d.
(-)-Limonene	n.d.

tr = traces; n.d. = enantiomer not evaluated due to low relative amounts (< 10%) in the essential oil

6.3 Use and scientific investigations

Essential properties are antidiabetic, neurotoxic, increasing blood pressure, decongesting the lymphatic system, anti-infectious, antiseptic, hyperemic, deodorant. The main indications are diabetes, burnout, hypotension, bronchitis, cough, sinusitis, asthma, arthritis, rheumatic polyarthritis, achy circulation, stiffness, inflammatory, and allergic processes (Zimmermann, 2018).

Genotoxic characteristics of the essential oils from stone pine needles were examined applying chromosome aberration and sister chromatids exchange tests in human lymphocytes *in vitro*, as well as *Drosophila melanogaster* somatic mutation and recombination test *in vivo*. Stone pine essential oil caused efficiently somatic mutations in *D. melanogaster*, but it was less genotoxic for human lymphocytes. The essential oils of stone pine needles caused chromosome aberrations at low toxicity levels (less than 20 % inhibition of miotic activity). The difference in the composition of the essential oil was responsible for the genotoxicity in various tests (Lazutka et al., 2001).

The essential oils of *P. sylvestris* showed high antimicrobial activity, a large efficacy against *Klebsiella pneumonia* isolated from nasal swab (MIC = 1.25 mg/mL and MBC = 2.50 mg/mL), *S. aureus* and *E. coli* from throat swab (MIC = 1.25 mg/mL and MB = 5.00 mg/mL), *S. aureus* from nasal swab and sputum (MIC = 2.50 mg/mL and MBC = 5.00 mg/mL) and against *Morganella morganii* (MIC = MBC = 2.50 mg/mL). There were no important variations in sensitivity between Gram-positive and Gram-negative strains, counting cell wall composition (Tabel 12). Oxygenated terpenes are known for antimicrobial potential. The highest antimicrobial efficiency of *P. sylvestris* essential oil might be connected to the high concentration of oxygenated terpenes such as α-murrolol, α-cadinol, and 13-epi-manool oxide. It was also proved that the antimicrobial activity of essential oils depends on multiple interactions between their compounds, which exhibit additive, synergistic, or antagonistic outcomes (Mitić et al., 2018).

Table 12. Antimicrobial activity of needle essential oils and referent antibiotic (MIC/MBC in mg/mL) (Mitić et al., 2018)

Isolated bacterial strains and their source	**Essential oils *P. sylvestris* MIC(mg/mL)**	**Essential oils P. sylvestris MBC(mg/mL)**
Klebsiella pneumoniae (nasal swab)	1.25	2.50
Klebsiella pneumonia (throat swab)	10.00	20.00
Escherichia coli (nasal swab)	10.00	40.00
Escherichia coli (throat swab)	1.25	5.00
Escherichia coli (sputum)	10.00	20.00
Morganella morganii (nasal swab)	2.50	2.50
Staphylococcus aureus (nasal swab)	2.50	5.00
Staphylococcus aureus (throat swab)	1.25	5.00
Staphylococcus aureus (sputum)	2.50	5.00

The essential oil of *P. sylvestris* showed the antifungal activity. The essential pine oil manifested a high inhibitory action against the growth of six tested *Cryptococcus neoformans* isolates to azole drugs (fluconazole, itraconazole, and voriconazole), and with low MIC values ranging from 0.07 to 0.27 mg/mL. Among the main components, carvacrol and α-pinene were especially useful in inhibiting the growth of all *C. neoformans* isolates. Terpinen-4-ol exhibited moderate antifungal activity, but γ-terpinene showed weak inhibition. By checkerboard testing and isobologram forms (concave), combinations of itraconazole with essential pine oils and carvacrol were discovered to be synergistic (the fractional inhibitory concentration index = FICI ≤ 0.5) against azole susceptible *C. neoformans* (Scalas et al., 2018).

Pinus sylvestris essential oil also showed insect larvicidal activity. The highest mortality was noticed for larvae and adults of *Dorsophila melanogaster* with an evaluated LC_{50} of 2.78 % at the end of the experiment (14 days) with a 95 % confidence interval of 2.15 – 3.42 % (Table 13). The species with a higher content of pinenes demonstrated topmost larvicidal action. The toxicity of essential oils against insects is associated with the presence of terpene and terpene-like compounds, as a natural defence against insects. *Pinus sylvestris* has a low content of pinene, but shows high toxicity. The high toxicity can be explained by synergistic interactions with oxygenated terpenes such as α-murrolol, α-cadinol, and 3-epi-manool oxide in stone pine (Mitić et al., 2018).

Table 13. Survival of *D. melanogaster* and developmental time (DT) after exposure to various concentrations of *P. sylvestris* essential oils (Mitić et al., 2018)

Isolated bacterial strains and their source	**Essential oils *P. sylvestris* MIC(mg/mL)**	**Essential oils P. sylvestris MBC(mg/mL)**
Klebsiella pneumoniae (nasal swab)	1.25	2.50
Klebsiella pneumonia (throat swab)	10.00	20.00
Escherichia coli (nasal swab)	10.00	40.00
Escherichia coli (throat swab)	1.25	5.00
Escherichia coli (sputum)	10.00	20.00
Morganella morganii (nasal swab)	2.50	2.50
Staphylococcus aureus (nasal swab)	2.50	5.00
Staphylococcus aureus (throat swab)	1.25	5.00
Staphylococcus aureus (sputum)	2.50	5.00

The essential oils of *P. sylvestris* showed a low antioxidant capacity. The antioxidant activities of the twigs and needles of five turkish Pinus species: *P. brutia*, *P. halepensis*, *P. nigra*, *P. pinea*, and *P. sylvestris* were tested using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and N, N-dimethyl-p phenylendiamine (DMPD) radical scavenging, metal-chelation, and ferric-reducing antioxidant power (FRAP) assays. The essential oils were tested in four *in vitro* assays for evaluation of their antioxidant potentials at 250, 500, and 1000 $\mu g/mL^{-1}$ concentrations. All of the essential oils were inactive against DMPD radical, but they had DPPH scavenging effect below 10 %. FRAP of Scots pine twigs essential oils was 0.255 ± 0.018 $\mu g/mL^{-1}$, of needles essential oils was 0.339 ± 0.037 at 1000 $\mu g/mL^{-1}$. In a metal chelating capacity test, the essential oil of *P. sylvestris* twigs was the most active one, with FRAP values 72.46 ± 3.72 %. These results revealed that *P. sylvestris* as the other Pinus species had antioxidative properties (Ustun et al., 2012). In the study from Kosovo, the antioxidant capacity of essential oil of *P. sylvestris* and four other Pinus species was also tested using the DPPH assay. *Pinus sylvestris* essential oil showed moderate antioxidant activity (0.224 ± 0.011 µg trolox equivalent / mL essential oil). F1 fractions eluted with hexane showed mainly non-oxygenated monoterpenes such as α-pinene, β-pinene, myrcene, limonene, β-caryophyllene, and D-germacrene, and the lowest value of the antioxidant activity in fraction F1 showed *P. sylvestris* with (F1 = 0.021 ± 0.01 µg trolox equivalent / mL essential oil). F2 fraction eluted with hexane/diethyl ether contained oxygenated monoterpenes and sesquiterpenes (esters, alcohols, oxides, ketones, and aldehydes). F2 was the most active fraction for all five Pinus species. The antioxidant activity of *P. sylvestris* in fraction F2 was 0.192 ± 0.02 µg trolox equivalent / mL essential oil. Fraction F3 was eluted with diethyl ether and included mainly alcoholic compounds, for example α-terpineol and borneol. The antioxidant activity of *P. sylvestris* fraction F3 was 0.104 ± 0.01 µg trolox equivalent / mL essential oil (Table 14). The components capable of the antioxidant activity in F2 were oxygenated monoterpenes and sesquiterpenes, which may act as radical scavenging agents. F3 showed lower antioxidant activity for all essential oils compared to F2, although they contained a high percentage of oxygenated monoterpenes. These results strongly indicate that the diffference in antioxidant activity of the essential oils should not be based on differences in their phytochemical profiles

alone. The potential synergistic or antagonist interactions among compounds should also be considered (Kurti et al., 2019).

Table 14. Antioxidant activity (DPPH values) of the essential oils (EO) and their chromatographic fractions. TE: trolox equivalent; Total EO: unfractioned EO; F1: n-hexane fraction; F2: n-hexane/diethyl ether (1:1) fraction; F3: diethyl ether fraction; ΣF1-F3: arithmetical sum of fractions DPPH values. SD: standard deviation (Kurti et al., 2019)

	DPPH value (μgTE/mL $_{EO}$ ± SD) from *P. sylvestris*
Total essential oil	0.224 ± 0.01
Fraction 1 (F1)	0.021 ± 0.01
Fraction 2 (F2)	0.192 ± 0.02
Fraction 3 (F3)	0.104 ± 0.01
ΣF1-F3	0.317 ± 0.02

A study from Turkey investigated Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) inhibitory activity (inhibition ± S.E.M. %) of the essential oils of the Pinus species and a reference substance (galanthamine) at 200 μg/mL^{-1} (Ustuna et al., 2012). Cholinesterase is the enzyme that catalyzes the hydrolysis of acetylcholine. The inhibition of cholinesterase can be useful as the treatment of Alzheimer's disease. There are two different cholinesterase enzymes, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). AChE has stronger acetylcholine hydrolytic activity than BChE under the same condition. AChE is substrate-specific and located in the brain in high concentrations, but BChE is nonspecific and spread everywhere in the body (Atatreh et al., 2019). The essential oils of *P. brutia*, *P. halepensis*, *P. nigra*, *P. pinea*, and *P. sylvestris* and pycnogenol (the bark extract of *P. pinaster*) showed a cholinesterase inhibitory potential. In the study, *P. halepensis* exhibited the most encouraging results, but *P. sylvestris* results should also be taken into consideration. The main identified components of the *P. halapensis* twigs essential oil, which showed highest efficacy against both cholinesterase, were β-pinene (18.7 %), limonene (18.7 %), and α-pinene (16.4 %). The cholinesterase inhibitory properties of α- and β-pinene are well-known, but limonene was also defined as an uncompetitive inhibitor of AChE. The amount of α- and β-pinene, and limonene in the *P. halapensis* essential oils could be the major reason for the occurrence of strong AChE and BChE inhibition. The high concentration of α- and β-pinene in *P. sylvestris* could be an explanation for moderate cholinesterase inhibitory potential (Ustuna et al., 2012). The

essential oils of Pinus species can be considered as the future therapy of Alzheimer's disease (Atatreh et al., 2019).

In an investigation from south-eastern Morocco (Tafilalet region) medicinal plants were used in the treatment of diabetes mellitus, hypertension, and cardiac diseases. Seven hundred persons were interviewed, including 320 diabetic patients and 380 patients with hypertension and cardiac disorders. 80 % of interviewed patients used medicinal plants to treat their disorders. They stated that phytotherapy was cheaper (58 %), more efficient (40 %), and better (65 %) than conventional medicine. In this ethnobotanical inquiry, about 92 medicinal plants were cited. The essential oils of Scots pine were used in the therapy of hypertension and cardiac diseases (Eddouks et al., 2002).

Another study investigated the effect of a cream with several essential oils on adjuvant arthritis (an experimental model of human rheumatoid arthritis) in Lewis rats. One group of rats received a cream with 20 % of essential oil topicaly twice a day for seven days, and the other group a placebo. The essential oils contained in the creme were from basil, bitter orange, black pepper, clary sage, cedarwood, clove bud, eucalyptus, foraha, fennel, ginger, immortel, lavender, nutmeg, Scots pine needle, rosemary, and sage. Pro-inflammatory cytokines TNF-α and IL-1β analysis of the synovial fluid and synovium infiltrating cells of rats showed notable suppression of TNF-α and IL-1β in animals treated with the cream with 20 % of essential oil in comparison to the placebo group of animals. The gelatin zymogram of the synovial fluid and synovium-infiltrating cells lysate revealed that matrix metalloproteinases (MMP) activity were also suppressed in rats treated with cream containing essential oils compared with placebo-treated rats. The levels of anti-mycobacterial heat-shock protein 65 (anti-Bhsp65) antibodies were unchanged by treatment. The results indicated that arthritic rats, which were treated for seven days with the essential oil containing cream showed an optimal reduction in arthritic inflammation of hind paws, and bone and cartilage damage of the paw joints. The placebo used in the study had no effect on the frequency or severity of adjuvant arthritis, so it shows that essential oil is the active antiarthritic component of tested cream. After seven days, when treatment was stopped, rats treated with the ointment containing 20 % of essential oil continued to improve (Figure 9) (Komeh-Nkrumah et al., 2012).

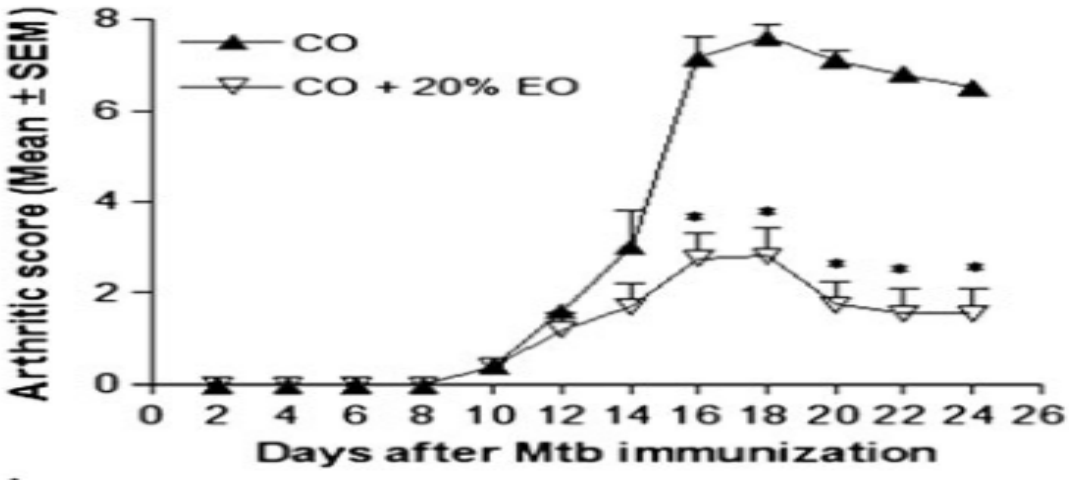


Figure 9. Comparison of the severity of arthritis in rats treated with ointment containing 20 % EO (essential oils) versus those treated with ointment lacking EO (Komeh-Nkrumah et al., 2012)

The study examined the potential cytotoxicity of Scots pine native in the European region. The cytotoxic activity of methanol extract and essential oil of Scots pine needles was tested in different human cancer cell lines. This needle extract, as well as essential oil of Scots pine needles, revealed cytotoxic activity to breast cancer cell lines. The cytotoxic activity of Scots pine extract and essential oil was compared on estrogen receptor (ER)-positive MCF7 and estrogen receptor (ER)-negative MDA-MB-231. Tamoxifen citrate and ellipticine were used as a positive control. The Scots pine essential oil manifested stronger cytotoxic effects on both breast cancer cell lines than the extract. IC_{50} values of oil were 28.67 and 29.23 µg/mL, respectively, but 80.20 and 42.27 µg/mL for the extract studied. The Scots pine needles essential oil, and the extract seems to be a potential chemopreventive or chemotherapeutic factor for breast cancer unresponsive to endocrine treatment (Table 15) (Hoai et al., 2015).

Table 15. Cytotoxic activity of Scots pine needles extract and essential oil on MCF-7 and MDA-MB-231 (Hoai et al., 2015)

Concentration (µg/ml)	Extract (IC_{50} µg/mL)	Essential oil (IC_{50} µg/mL)	Tamoxifen Citrate (IC_{50} µg/mL)	Ellipticine* (IC_{50} µg/mL)
MCF-7				
100	58.41	101.87	98.11	84.99
20	18.83	39.60	84.38	71.94
4	8.75	18.42	30.26	45.66
0,8	2.61	5.24	− 8.49	15.58
IC_{50}	80.20	28.67	8.66	0.50
MDA-MB-231				
100	6.,39	97.58	101.00	86.06
20	35.72	41.86	99.79	76.07
4	18.01	18.53	17.13	42.50
0,8	3.04	12.67	1.07	11.24
IC_{50}	42.27	29.23	5.57	0.55

*The concentrations of ellipticine in those experiment were 10 µg/mL; 2 µg/mL; 0.4 µg/mL and 0.08 µg/mL. IC_{50}: Half maximal inhibitory concentration; MDA-MB-231

7 *Pinus nigra* J.F.Arnold

7.1 General

Pinus nigra J.F.Arnold is known as Austrian pine or black pine. There are five subspecies and one variety recognized*: P. nigra subsp. nigra*, *P. nigra subsp. dalmatica*, *P. nigra subsp. laricio*, *P. nigra subsp. pallasiana var. pallasiana*, and *P. nigra subsp. pallasiana var. fastigiata*. It is widespread in south-west, south and south-east Europe, north Algeria, north Marocco, Cypreus and from Krym along Black Sea to Krasnodor in Caucaus.

Black pine is up to 40 m tall and up to 1.89 m wide. Leaves are in fascicles of two, 8 - 16 cm long and 1 - 2 mm wide, straight or more curved, rigid or flexible. Seed cones are ovoid-conical, 5 - 10 cm long and 2 - 4 cm wide (Farjon, 2010d).

7.2 Composition of essential oils

The identification of essential oils of *P. nigra* was performed by GC, GC-FID, GC–MS and ^{13}C-NMR. Fresh needles were collected from wild-growing populations from the central Balkan (Zmajevački potok and Jarešnik), coastal regions of Croatia (on the Dalmatian islands) and different locations in Corsica (Table 16).

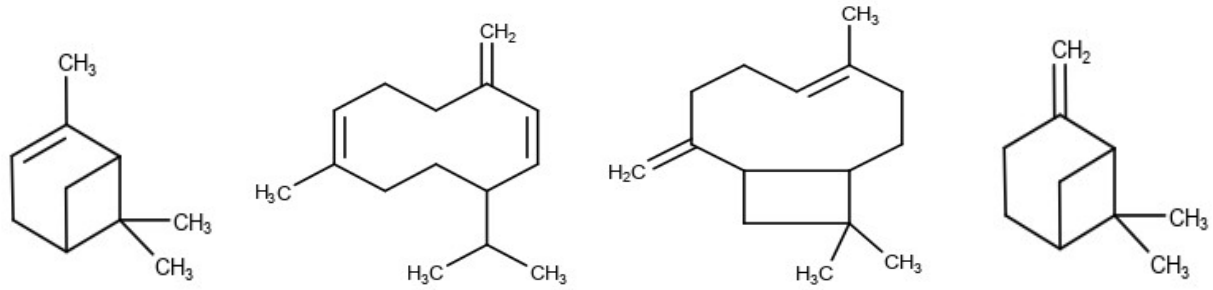
The major components in the essential oils of needles of *P. nigra* were α-pinene, germacrene D, (E)-caryophyllene and β-pinene (Figure 10). These four terpene components were also found in the needle oils of black pine from Turkey (ssp. *pallasiana*), the Dalmatian islands (ssp. *dalmatica*), and Corsica (ssp. *laricio*) (Šarac et al., 2014).

Tabel 16. Chemical composition of the essential oils of *Pinus nigra* from different locations

Compounds	**Twigs with needles from *P. nigra subsp. nigra*** (Zmajevački potok) (Šarac et al., 2014)	**Needles of *P. nigra subsp. dalmatica*** (Dalmatian islands) *(*Politeo et al., 2011)	**Needles of *P. nigra subsp. laricio*** (Corsica) (Rezzi et al., 2001)	**Twigs with needles from *P. nigra subsp. pallasiana*** (Jarešnik) (Šarac et al., 2014)
Tricyclene	0.16			0.40
α-Thujene	0.41			0.41
α-Pinene	45.93	24.4	41.0	42.33
Camphene	0.95	1.5	1.0	1.95
Sabinene	0.31			0.26
β-Pinene	6.90	16.0	3.5	5.15
Myrcene	0.88	1.9	7.7	0.45
α-Phellandrene	0.00		0.2	0.00
ß-Phellandrene		1.9	3.7	
Δ3-Carene	0.03			0.70
Limonene	2.16	3.3	5.1	3.08
(E)-β-Ocimene	0.86	2.1	2.8	0.27
γ-Terpinene	0.00		0.1	0.00
Terpinolene	0.57	1.4	0.5	0.20
trans-Verbenol	0.00			0.01
α-Terpineol	0.00	1.9	1.3	0.00
Linalool		0.3	1.8	
Linalool acetate	0.05	0.4	1.2	0.04
Bornyl acetate	0.63	3.3	0.3	0.62
trans-Verbenylacetate	0.03			0.01
trans-Pinocarvyl acetate	0.01	0.6		0.00
Trans-verbenol		0.2		
Myrtenyl acetate	0.03			0.00
δ-Elemene	0.00			0.02
α-Terpinylacetate	0.48	2.9	0.9	0.14
α-Cubebene	0.00			0.01
α-Copaene	0.02			0.03
Geranyl acetate	0.00			0.02
β-Bourbonene	0.02			0.04
β-Cubebene	0.00			0.04
β-Elemene	0.03	0.6		0.01
Thymyl methyl oxide			0.4	
(E)-β-Caryophyllene	8.13	9.6	5.3	7.43

Table 16 continued				
Terpinen-4-ol		0.641	0.2	
β-Copaene	0.02			0.10
6,9-Guaiadiene	0.00			0.02
Estragole		0.3	0.1	
α-Humulene	1.24	2.5	0.8	1.07
(E)-β-Farnesene	0.01			0.00
γ-Muurolene	0.34		0.8	0.37
Germacrene D	27.50	14.6	8.6	30.59
Germacrene B		0.3		
Geranyl acetateŁ			0.5	
β-Selinene	0.01			0.02
Phenylethyl 3-methylbutanoate	0.12			0.00
γ-Amorphene	0.15			0.00
Bicyclogermacrene	0.19			0.62
α-Muurolene	0.08	0.8		0.22
Germacrene A	0.09			0.05
γ-Cadinene	0.25		0.3	0.37
δ-Cadinene	0.61	2.1	1.3	0.69
Geraniol			0.2	
Germacrene D-4-ol	0.57			1.93
Phenylethyl isovalerate		2.3	1.3	
Caryophyllene oxide	0.08	0.3	0.2	0.04
1,7-Diepi-α-Cedrenal	0.00			0.06
T-Cadinol	0.03			0.01
α-Cadinol	0.03	0.4	0.4	0.02
Germacra4 (15), 5, 10 (14)-trien-1-alpha-ol	0.00			0.13
Methyl eugenol		0.4	0.1	
τ-Muurolol			0.2	
Oplopanone	0.02			0.00
Tetradecanoic acid		2.8		
Manoyl oxide			1.9	
13-*epi*-Manoyl oxide			0.2	
Unknown components	0.05			0.05

α-Pinene, *β*-pinene, limonene und camphene dominated in the levorotary more than in the dextrorotary forms (Table 17) (Ochocka et al., 2008).



α-pinene germacrene D (E)-caryophyllene β-pinene

Figure 10. Major volatile constituents of essential oils of *Pinus nigra*

Table 17. Enantiomeric ratio of the essential oil from *Pinus nigra* (Ochocka et al., 2008)

Compounds	Precentage (%)
(+)-α-Pinene	14.8
(-)-α-Pinene	72.6
(+)-β-Pinene	0.2
(-)-β-Pinene	2.8
(+)-Limonene	1.0
(-)-Limonene	3.2
(+)-Camphene	0.2
(-)-Camphene	3.3

7.3 Use and scientific investigations

Essential properties are antiseptic (room air), toning, stimulating, decongesting on the respiratory tract and the lymphatic system, and *P. nigra* is used as adjuvants for prostate hyperplasia. The main indications are burnout, bronchitis, sinusitis, prostatitis (Zimmermann, 2018).

The study investigated the antioxidant activity of turpentine excrete from *P. nigra subsp. pallsiana* (TPN). All of the doses (100, 300, and 500 µg) of TPN showed higher antioxidant activity than α-tocopherol (natural antioxidants). The results of the study demonstrated good antioxidant activity, the reducing power, superoxide anion radical scavenging, free radical scavenging, metal chelating, and hydrogen peroxide scavenging activities of TPN depending on concentration and raising with a raised level of TPN. The phenolic compounds of TPN seem to be responsible for the antioxidant

activity. The results of the study show that TPN can be used as a source of natural antioxidants (Figure 11) (Gülçin et al., 2003).

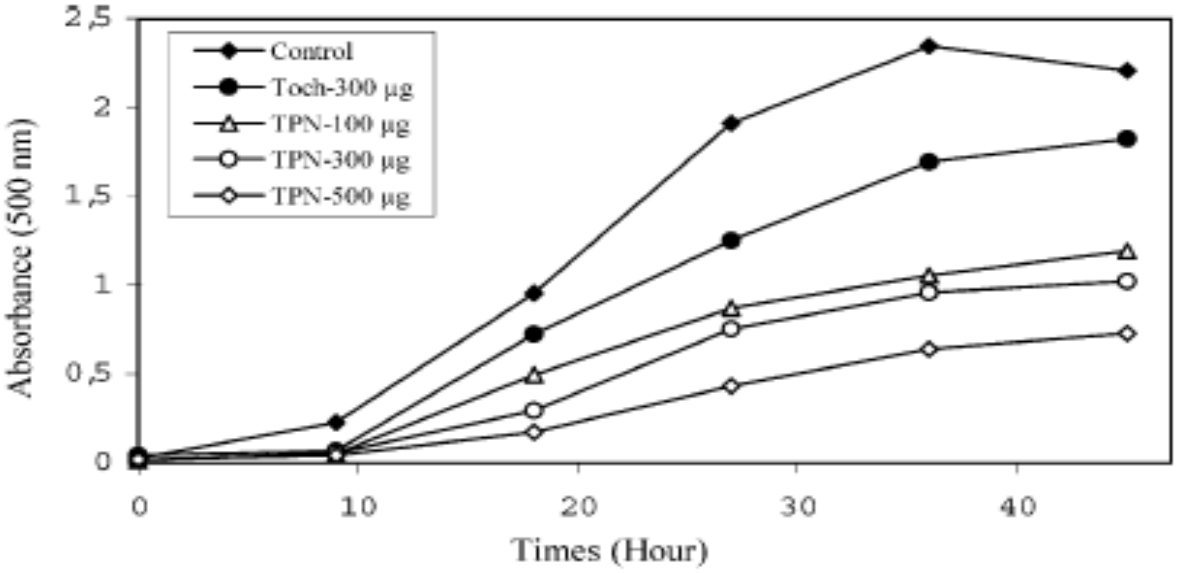


Figure 11. Total antioxidant activity of α-tocopherol and different doses of TPN in linoleic acid emulsion. TPN: turpentine of *Pinus nigra* Am. *Subsp. pallisiana* (Lamb.) Homboe; α-tocopherol; n=2 (Gülçin et al., 2003)

Comparison of DPPH radical scavenging method, Ferric Reducing Antioxidant Potential (FRAP), and Thiobarbituric Acid Reactive Species (TBARS) assays for evaluating the antioxidant activity of the essential oil from *P. nigra ssp. dalmatica* show low antioxidative potential. The oil radical scavenging activity tested by the DPPH (2,2-diphenyl-1-picrylhydrazyl) method was dose-dependent and low compared to commercial antioxidants. The low antioxidant activity of the essential oil of *P. nigra ssp. dalmatica* can be explained by a high concentration of monoterpene hydrocarbons that don´t have phenol groups. Phenol groups are necessary for a high radical scavenging effect (Figure 12) (Politeo et al., 2011).

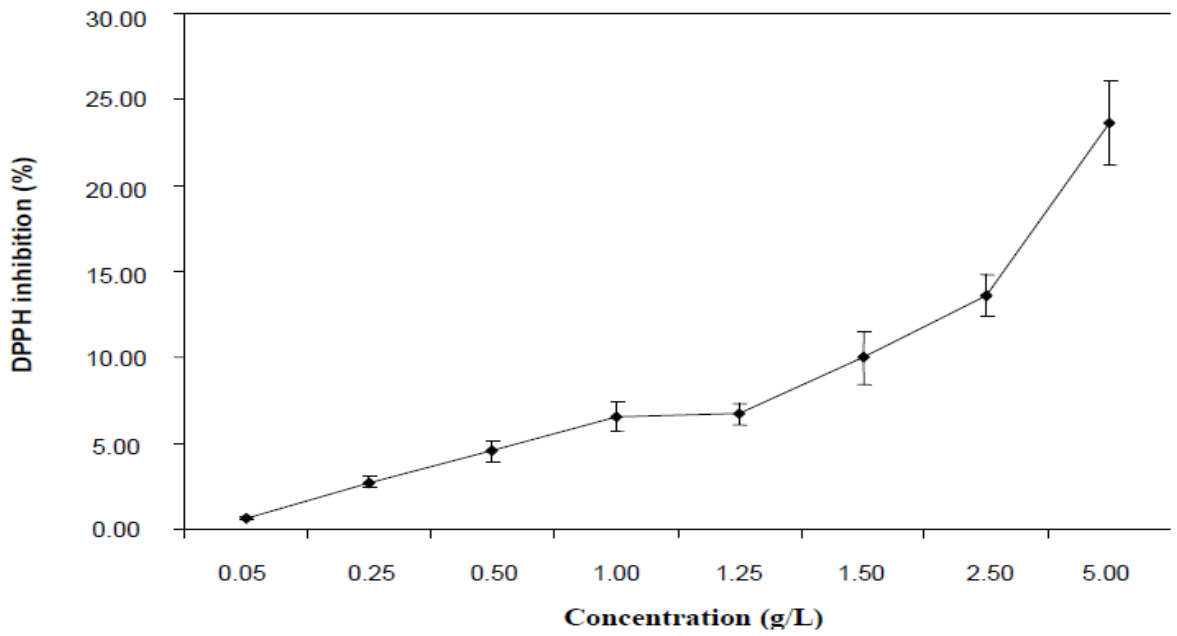


Figure 12. The DPPH radical scavenging activity in the presence of the *P. nigra* ssp. *dalmatica* essential oil (Politeo et al., 2011).

In vitro antimicrobial activity of the essential oil of three *P. nigra* subspecies was tested against bacterial (three Gram-positive and three Gram-negative) and fungal strains. Only one fungal (*Aspergillus niger*) and two bacterial strains (*S. aureus* and *Bacillus cereus*) gave a sensitivity against applied essential oils. *P. nigra ssp. nigra* essential oil showed the lowest antimicrobial activity. MIC of the *P. subsp. nigra* oil was in the concentration range from 2.50 to 20.00 mg/mL. The essential oil of *P. ssp. pallasiana* showed higher activity than the P. *ssp. nigra* with active inhibitory concentrations in the range from 1.50 to 20.00 mg/mL. The essential oils of the *P. nigra var. banatica* showed the highest antimicrobial activity, with concentrations ranging from 0.62 to 20.00 mg/mL. This oil was effective against all tested strains except against Gram-negative bacterium *E. coli*. The antimicrobial activity of the essential oil of *P. nigra ssp. dalmatica* was tested in another study and also represented antimicrobial and antifungal activity. The studies did not use the same set of strains, but it was possible to compare the results for some strains at the species level. The most sensitive strain was *B. cereus* with MIC in the range of 0.62 – 2.50 mg/mL. The most responsive strain in the study was *S. aureus* with MIC in range of 10–20.00 mg/mL. *Escherichia coli* manifested the highest resistance, where

concentrations above 20.00 mg/mL were necessary to inhibit its growth. Opposite results were in the case of fungal organisms, where *A. niger* showed higher susceptibility than *C. albicans* to the oil of *P. nigra*. Similarities, such as higher sensitivity of the Gram-positive strains and complete resistance of Gram-negative enterobacteria were noted (Table 18). Mono- and sesquiterpene hydrocarbons dominated the essential oils of *P. nigra*, which are known as volatile components with low antimicrobial activity due to their low hydrogen bonding capacity and water solubility. The essential oil of *P. nigra var. banatica* had the same main compounds as the other two oils, but it showed the highest antimicrobial activity. This could be explained by the synergistic effect of some minor components such as thujene, myrcene, and camphene, which were previously published as antimicrobials (Šarac et al., 2014).

Table 18. Minimum inhibitory activities of the *P. nigra ssp. nigra, P. nigra ssp. Pallasiana* and *P. nigra var. banatica* needle essential oils (Šarac et al., 2014)

Bacterial/fungal strain	***P. nigra ssp. nigra* mg/mL MIC**	***P. nigra ssp. pallasiana* mg/mL MIC**	***P. nigra var. banatica* mg/mL MIC**	**Positive control mg/mL**
Gram-positive enterobacteria				
Staphylococcus aureus	20.00	10.00	20.0	0.19[a]
Sarcina lutea	> 20.00	20.00	10.0	0.78[a]
Bacillus cereus	2.50	1.50	0.62	0.19[a]
Gram-negative enterobacteria				
Proteus mirabilis	> 20.00	> 20.00	20.0	0.04[a]
Esherichia coli	> 20.00	> 20.00	> 20.0	0.19[a]
Pseudomonas aeruginosa	> 20.00	20.00	10.0	0.19[a]
Fungi				
Candida albicans	> 20.00	10.00	5.0	0,9[b]
Aspergillus niger	10.00	5.00	5.0	1.9[b]

a = Chloramphenicol; b = Nystatin.

Pinus species were used in Turkish folk medicine against rheumatic pain and for wound healing. Wound healing and anti-inflammatory activities of the essential oils from the cones and needles of five different Pinus species (*P. brutia*, *P. halepensis*, *P. nigra*, *P. pinea* and *P. sylvestris*) were tested. Skin samples were histopathologically analyzed. The results demonstrated that the groups that were treated with *P. pinea*, *P. halepensis*, and *P. nigra* enriched creams had a strong re-modeling, especially reepithelization

effect. It could also be demonstrated that the essential oil of *P. nigra* and *P. pinea* were essential for anti-inflammatory activity, which was based on the increased inhibition of acetic acid-induced in capillary permeability (Table 19). α-Pinene was the major component in the essential oils of *P. halepensis*, *P. nigra* and *P. sylvestris*. *P. pinea* essential oils showed a higher amount of limonene and β-pinene. Limonene is well-known as an important monoterpene in the wound healing process and its high concentration in *P. pinea* could support the wound healing activity. Terpenic compounds showed bactericidal, fungicidal, insecticidal, pesticidal, antioxidant, anti-inflammatory, analgesic and sedative effects, which is very important for wound healing activity. They create a barrier against microbial attacks and preserve the wound from infection. The wound-healing effects of the pine essential oils could be connected to their antimicrobial activity (Süntara et al., 2012).

Table 19. Inhibitory effect of the essential oils from *Pinus sylvestris* on acetic acid-induced increased capillary permeability (Süntar et al., 2012)

Material	Parts used	Dose (mg/kg)	Evans blue concentration (µg/mL) ± SEM	Inhibition (%)
Control			8.93 ± 1.17	
Pinus nigra	Cones	100	8.44 ± 1.21	5.5
Pinus nigra	Cones	200	8.14 ± 1.12	8.9
Pinus nigra	Needles	100	7.99 ± 1.39	10.5
Pinus nigra	Needles	200	6.17 ± 0.78	30.9*
Indomethacin		10	3.99 ± 0.21	55.3**

S.E.M.: standard error mean; * $p < 0.05$ significant from the control; ** $p < 0.001$ significant from the control

Possible antioxidant and analgesic activities of turpentine excretes from *P. nigra* Arn. *subsp. pallsiana* (TPN) was investigated in a study. The total antioxidant activity was dose-dependent and raised with the increasing volume of extracts (100, 300, and 500 µg) added to a linoleic acid emulsion. TPN showed higher antioxidant activity in all doses than α-tocopherol (natural antioxidant = reference). TPN also had 49, 70, and 91 % inhibition on peroxidation of linoleic acid, respectively, better than α-tocopherol (40 %). The results indicated that TPN had strong antioxidant acitivity, reducing power, superoxide anion radical scavenging, free radical scavenging, metal chelating, hydrogen peroxide scavenging, and analgesic activities when compared with different standards such as α tocopherol, quercetin, BHA (butylated hydroxyanisole), BHT (butylated

hydroxytoluene), and metamizol. Phenolic compounds seem to be responsible for the antioxidant activity of TPN. The study showed a strong analgesic effect of TPN, which was compared with metamizol as a standard analgesic compound. It is assumed that there is a relationship between antioxidant and analgesic activities, but also that analgesic activity may be related to its antioxidant activity (Gülçin et al., 2003).

Herbicidal effects of the black pine oil on weed germination and seedling growth were investigated and proven in one study. The phytotoxic effects of *P. nigra* oil were tested on *Phalaris canariensis* L., *Trifolium campestre* Schreb. and *Sinapis arvensis* L. (common weeds in Tunisia). The inhibition of germination and seedling growth of weeds was dose dependent. High concentration of the oil (5 $\mu L/ml^{-1}$) completely inhibited germination and seedling growth of weeds (Amri et al., 2014).

The essential oils of *Pinaceae* showed antidementia effect by inhibiting enzyms of cholinesterase. The AChE and BChE inhibitory activity of the essential oils from *P. nigra subsp. nigra*, *P. nigra var. calabrica*, and *P. heldreichii subsp. leucodermis* were investigated. Since cholinesterase drugs are the only drugs available to treat Alzheimer's disease, this activity of *Pinaceae* essential oil is significant. All the tested oils were able to inhibit AChE and BChE in a dose-dependent manner. An effective action against AChE demonstrated *P. nigra subsp. nigra*, with IC_{50} values of 94.4 µg/mL. BChE looked less sensitive to *P. nigra subsp. nigra* essential oil, with IC_{50} values of 162.5 µg/mL. Previous studies reported that terpen rich essential oils exhibited an intense inhibitory activity against both enzymes. Five compounds, particularly β-phellandrene, terpinolene, terpinen-4-ol, linalylacetate, and trans-caryophyllene, were tested to evaluate their own activity. β-Phellandrene was selective against AChE, with IC_{50} value of 120.2 µg/mL, while trepinolene inhibited both AChE and BChE enzymes with IC_{50} values of 156.4 µg/mL and 147.1 µg/mL, respectively. Other tested terpenes manifested BChE inhibitory activity, with IC_{50} values ranging from 78.6 µg/mL for trans-caryophyllene to 168.7 µg/mL for linalyl acetate (Table 20). The results showed that Pinus essential oils and some constituents have the ability to treat Alzheimer's disease (Bonesi et al., 2010).

Table 20. Cholinesterase inhibitory activity of essential oils and identified constituents from Pinus species (IC_{50}, µg/mL) (Bonesi et al., 2010)

	Acetylcholinesterase (AChE)	Butyrylcholinesterase (BChE)	SI (BChE/AChE)
Essential oils			
P. heldreichii subsp. *leucodermis*	51.1 ± 1.8*	80.6 ± 2.3*	1.6
P. nigra var. *calabrica*	101.5 ± 2.4*	128.0 ± 2.4*	1.3
P. nigra subsp. *nigra*	94.4 ± 1.8*	162.5 ± 1.4*	1.7
Terpenes			
β-Phellandrene	120.2 ± 2.0*	> 50	
Terpinolene	156.4 ± 2.8*	147.1 ± 2.5*	0.9
Terpinen-4-ol	21.4 % (1.2 mM)[21]	107.6 ± 1.2*	
Linalyl acetate	38 % (82 µg/ml)[35]	168.7 ± 2.1*	
trans-Caryophyllene	32 % (0.06 mM)[22]	78.6 ± 1.3*	
Positive controls			
Physostigmine	0.1 ± 0.01	0.2 ± 0.01	2
Galanthamine hydrobromide	0.3 ± 0.04	3.0 ± 0.04	10

Note. IC_{50} values are mean ± SD (n = 3). One-way ANOVA analysis: ***p<0.0001; Dunnett's test: *p<0.01

8 Conclusion

The use of pine essential oil is medicinal, pharmaceutical, and cosmetic. It can be inhaled, applied orally or topically. Taken orally, it supports immune function by eliminating harmful bacteria and fungus. It can be considerd as antidementiva, because of its cholinesterase inhibitory activity. When inhaled, essential pine oils reduce stress, enhance concentration, clear the respiratory tract and calm symptoms of cold, cough, sinusitis, asthma, flu, and help easier breathing. Topically used pine essential oil helps to calm skin thanks to its antiseptic and antimicrobial properties. Its antioxidant properties are ideal for use to slow the appearance of signs of aging. The circulation-stimulating property of pine oils promote a warming effect. That is good for calming muscles and joints, that may be caused by arthritis and rheumatism. The efficacy of essential pine oil is dependent on its composition. Some compounds showed remarkable effect when being isolated from essential pine oil, such as α-pinene, β-pinene, ∆3-carene and limonene. The implementation of essential pine oil has a long history, but it also has many new activities that yet have to be investigated and proved.

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