The Pharmaceutical and Chemical Journal, 2016, 3(2):91-98

Available online www.tpcj.org

Research Article

ISSN: 2349-7092
CODEN(USA): PCJHBA

Chemical Composition and Evaluation of Antimicrobial Activity of Essential Oils Isolated from *Achillea kotschyi* Boiss. subsp. *kotschyi* (Asteraceae) of Lebanon

Mneimne Malek[1], Baydoun Safa [2], Nemer Nabil[1], Arnold Apostolides Nelly[1]

[1]Faculty of Agriculture and Food Sciences, USEK, Kaslik, Lebanon.
[2]Research Center for Environment and Development, Beirut Arab University, Lebanon.

Abstract Essential oils of the flowers and fruits *of Achillea kotschyi* Boiss. subsp. *kotschyi* (Asteraceae) growing wild in Lebanon were, for the first time, screened for their antimicrobial inhibitory activity. The composition of the essential oils was analyzed by GC and GC-MS; 37 compounds and 14 compounds representing 68.3%, and 95.5% of total oils, were found in the essential oils of flowers and fruits, respectively.
Also, nerolidol 2 (14%), β-terpinene (13.6%), β-myrcene (9.2%), trans-β-farnesene (4.1%), α-bisabolol (3.2%), α-farnesene (3.2%), α-phellandrene (3.2%), and piperitone (3.2%) were the major components found in the extracted essential oil of flowers. Whereas, dominance of β-thujone (58.5%) followed by 1,8-cineole (8.3%), camphene (7.3%), β-selinenol (5%), sabinene (3.7%), α-pinene (2.8 %) and α-selinene (2.8%) characterized the fresh fruits essential oil.
The essential oils showed significant inhibitory antibacterial and antifungal activity mainly on Gram negative *S. enteritidis* and both Gram positive *S. aureus* and *E. faecalis* as well as the fungus *A. fumigatus*. The results of the study showed an interesting antimicrobial profile which could provide promising pharmaceutical and economical benefits of the potential use of the plant essential oils.

Keywords Achillea kotschyi; essential oil; β-thujone; nerolidol 2; β-terpinene; β-myrcene; 1,8-cineole; antimicrobial.

Introduction

The genus *Achillea* (Asteraceae) represented by more than 140 perennial herbaceous species is widespread in Southern Europe, Mediterranean and Middle East region. Commonly known as Yarrow, the genus was named after Achilles of the Greek myth Iliad for his use of the plant for wound healing during the Trojan war [1]. Nine *Achillea* species grow in Lebanon, four of which (i.e. *A. falcata*, *A. kotschyi*, *A. fragrantissima*; *A. membranacea*) are native to East Mediterranean region [2-3].
Since antiquity, *Achillea* species are traditionally used in a wide range of medicinal indications. They are generally, known as remedies for wound healing and as antimicrobial, antioxidant and anticancerous [4-8]. The species are also shown to exhibit strong antimicrobial properties against some food-borne bacteria, fungi and yeast [9] and insecticidal activities [10-11].
Studies on *Achillea* sp. revealed highly variegated patterns of chemical composition. The main compounds of several species growing in Turkey were mostly 1,8-cineole, *p*-cymene, viridiflorol, nonacosane, α-bisabolol, caryophyllene oxide, α-bisabolon oxide A, β-eudesmol, 15-hexadecanolide, camphor [12]. Whereas, camphor, spathulenol, 1,8-cineole, salvial-4 (14)-en-1-one, eudesm-4-en-6-one, caryophyllene oxide and filifolone characterized some species from Iran [13], while some oils showed 1,8-cineole, borneol, camphor [14], *β*-pinene, 1,8-cineole, E-caryophyllene, germacrene D, chamazulene [15], artemisia ketone, camphene, 1-methyl-2-(1-methylethenyl), grandisol and fragranol [16-17], and 1,8-cineole, 4-terpineol, *trans*-carveol *cis*-ascaridole, *p*-cymene, carvenone oxide and camphor as the main components [18].

Achillea kotschyi Boiss. subsp. *kotschyi*, known by the local communities as "Hachichet El Jereh" or "Ghessoum" or "Ekliyyet Kitschi", is a native species that grows wild in Lebanon. The plant is most commonly used against stomach complaints and for the treatment of wounds and diabetes. It has been listed as a Rare Vulnerable species in 1997 IUCN Red List of Threatened Plants [19] and even Critically Endangered in Bulgaria (e-ecodb.bas.bg/rdb/en/vol1/Achkotsc.html.).

Although *A. kotschyi* subsp. *kotschyi* is distributed in Turkey, Greece, Bulgaria and Lebanon, limited studies on chemical constituents and bioactivity of the species have, to date, been reported. The main constituents of *A. kotschyi* growing in Turkey were reported as 1,8-cineole (22.5%), caryophyllene oxide (10.1%), *p*-cymene (8.4%) and hexadecanoic acid (7.7%) [20]. The antimicrobial activity of alcoholic extract of its flower heads was shown as moderate in comparison to other species [21]. However, the species was recently indicated to have a very high wound healing activity by stimulating collagen synthesis and fibroblast migration [4]. Investigations on the species growing in other regions are considered necessary to further elucidate the properties and potential activities of the plant. To our knowledge, no studies have been, to date, conducted on the plant growing wild in Lebanon. The present study concerns the chemical composition and antimicrobial activity of the essential oil of this Lebanese plant.

Material and Methods

Plant material

Aerial parts of the wild growing *Achillea kotschyi* were collected from Tannourine in the North of Lebanon in July 2014, at 1750 meters. The species identification was performed using the determination keys of the New Flora of Lebanon and Syria [3]. A voucher specimen (RCED2015-260) was deposited at the herbarium of the Research Center for Environment and Development, Beirut, Arab University, Lebanon.

Essential oil isolation

The essential oil of fresh parts (leaves, flowers and fruits) was hydrodistillled by Clevenger-type apparatus for three hours (European Pharmacopeia, 2008). The oil was dried overnight using anhydrous sodium sulphate and then stored in sterile sealed vials in the dark at 4ºC until analysis time.

Bacterial and fungal strains

Certified bacterial and fungal strains (Medi Mark, Europe) were used in screening the antimicrobial potency of *A.kotschyi* subsp. *kotschyi* essential oils. They were four pathogenic bacteria *Enterococcus faecalis* ATCC 29212 (Gram positive), *Staphylococcus aureus* ATCC 25923 (Gram positive), *Escherichia coli* ATCC 8739 (Gram negative), *Salmonella enteritidis* ATCC 13076 (Gram negative), and 3 certified pathogenic fungi; *Aspergillus fumigatus* ATCC 1022, *Candida albicans* ATCC 10231, *Trichophyton mentagrophytes* ATCC 9533.

GC and GC-MS analyses

GC and GC-MS analyses of the oils were performed by Agilent Technologies 7890 gas chromatography equipped with a Flame Ionization Detector (FID) and a HP- 5 MS 5% capillary column (30m x 0.25mm x 0.25µm film thickness). Mass spectra were recorded at 70 eV of electron energy and a mass range of 50-550 m/z. The carrier gas was Helium at a flow of 0.8 ml/min. The initial column temperature was 60ºC programmed to increase to 280ºC at a rate of 4ºC/min. The split ration was 1:40. The injector temperature was set at 300ºC. The purity of helium gas was 99.99%. A sample of 1 ml was injected manually in the split mode. Components identification was based on retention indices and comparison with mass spectral data of authentic standards and computer matching with Wiley 229, NIST 107, NIST 21 libraries as well as by comparing the fragmentation patterns of the mass spectra with those reported in the literature.

Antimicrobial activity by disc diffusion method

The antibacterial and antifungal activity of essential oil was carried out by disc diffusion method using 100µl of suspension containing 10^6 CFU/ml of microorganisms was spread on Muller-Hinton agar medium (Merck). Sterile 6 mm diameter filter paper discs (Whatman No. 3) were impregnated with 10 µl of essential oil and were placed on the agar. Standard reference discs of the antibiotics Norfloxacin (10 µg) and Nystatin (100 µg) were used as standard antimicrobial positive controls. Each test was run in triplicate and the mean values ±SD were considered. A blank disc was used as a negative control. The bacterial cultures were incubated at 37ºC for 24 hrs. Whereas *Candida albicans* and *Trichophyton mentagrophytes* were incubated at 27ºC for 48 hrs and 5 days, respectively. The diameters of growth inhibition zones around discs were measured using a caliper.

Determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration by Agar Dilution Method

MICs were determined by agar dilution method approved by NCCLS (1997) [22]. A series of four concentrations of oil (5, 10, 25 and 50 µl) with 0·5% (v/v) Tween-20 were added to one ml of microbial suspension containing approximately 10^6 CFU/ml of each organism in Muller Hinton broth. Tween-20 (Sigma) was used to enhance the solubility of essential oil in broth. 100 µl of each mixture was spread on Mueller Hinton agar plates. The plates inoculated with bacteria were incubated at 37 °C for 24 hrs and those inoculated with *Candida albicans* and *Trichophyton mentagrophytes* were incubated at 27°C for 48 hrs and 5 days respectively. MICs were determined as the lowest concentration of oil inhibiting the visible growth of each microorganism on the agar plate and MBC or MFC was taken as the concentration that completely kill the tested bacteria or fungal strains. The presence of one or two colonies was not considered. All tests were performed in triplicates.

Results and Discussion

Composition of Essential Oil

The essential oil yield obtained of studied parts of *A. kotschyi* Boiss. subsp. *kotschyi* was 0.73% in flowers and 0.91% in fruits. No detectable amounts were observed in either the stems or leaves. The peculiar result of the leaves is in contradiction with the general consensus that leaves are generally the plant parts that contain relatively highamount of essential oil [23]. According to comprehensive data, essential oils of *Achillea* species are significantly influenced by several biotic and abiotic and that major differences could be found between the vegetative organs and the phenological phases before and after flower development [24].

The GC and GC–MS analyses of essential oils revealed the identification of 37compounds in the flowers essential oilrepresenting 68.3% of total oil and 14compounds of the fruits essential oil representing 95.5% of total oil (Table 1). It is clearly noted that the composition pattern of the volatile oils totally varied between flowers and fruits and displayed different proportions of monoterpene hydrocarbons (33.4% and 14.7%, respectively), oxygenated monoterpenes (4.0% and 71.4%, respectively), sesquiterpene hydrocarbons (13.3% and 4.2%, respectively) and oxygenated sesquiterpenes (17.6% and 5.2%, respectively). Nerolidol 2 (14%), β-terpinene (13.6%), β-myrcene (9.2%), trans- β-farnesene (4.1%), piperitone (3.2%), α-farnesene (3.2%), α-bisabolol (3.2%), and α-phellandrene (3.2%) were the main compounds. Whereas, remarkable dominance of β-thujone (58.5%) followed by 1.8-cineole (8.3%), camphene (7.3%), β-selinenol (5.0%), α-selinene (2.8%), α-pinene (2.8%) and several minor components characterized the fruit oil. Similar characteristic qualitative and quantitative variations in the composition of the essential oils distilled from different plant organs have also been recognized in several studies. Both compound groups in the oils of flowers and fruits and sabinene being found in both (0.8% and 3.7%, respectively) were not reported among the main compounds of *A. kotschyi* subsp. *kotschyi* of Turkey. In the study, the plant was characterized by 1.8-cineole (22.5%), caryophyllene oxide (10.1%), *p*-cymene (8.4%), hexadecanoic acid (7.7%), β-eudesmol (4.9%), viridiflorol (3.9%), camphor (2.8%), spathulenol (2.6%) and caryophylladienol II (2.4%) as being the main constituents which, with the exception to 1.8-cineole, were not recorded in the plant herein studied [20]. It may be noteworthy to highlight the pronounced level of β-thujone (58.5%) and associated potential toxicity of the fruit oil of the plant [25-26]. These findings strongly confirm the substantial variations in oil compositions of the plant growing under different ecogeographical and environmental conditions.

Table 1: Chemical composition of the essential oils of *A. kotschyi* subsp. *kotschyi* growing wild in Lebanon.

Retention Time	Compound	Content %	
		Flowers	Fruits
7.51	1R-α-Pinene	0.5	-
7.91	Camphene	0.1	-
8.62	Sabinene	0.8	-
8.86	Sabinene	-	3.7
9.16	β-Myrcene	9.2	-
9.51	α-Phellandrene	3.2	-
9.88	δ-3-Carene	0.4	-
10.11	α-Terpinene	-	0.5
10.26	β-Terpinene	13.6	-
10.54	β-Trans-ocimene	1.8	-
10.88	β-Cis-ocimene	1.9	-
11.53	Γ-Terpinene	-	0.4

14.32	Neo-allo-ocimene	1.7	-
15.79	Camphene	-	7.3
18.93	α-Pinene	-	2.8
41.19	M-Menth-3(8)-ene	0.2	-
10.64	1,8-Cineole	-	8.3
11.12	Piperitone	3.2	-
14.57	β -Thujone	-	58.5
17.02	Terpinen-4-ol	0.1	-
17.18	Borneol L	-	3.0
17.87	Terpinen-4-ol	-	1.0
18.96	A-Phellandrene epoxide	0.1	-
24.99	Piperitone	-	0.6
37.05	2-Dehydro-1,8-cineole	0.6	-
35.47	α-Cedrene	0.3	-
35.71	Caryophyllene	0.5	-
36.00	(+)-Epi-bicyclosesquiphellandrene	0.1	-
36.18	γ-Elemene	0.1	-
36.39	γ-Muurolene	0.1	-
36.45	δ-Cadinene	0.1	-
36.97	Trans-β-Farnesene	4.1	-
37.16	γ-Cadinene	0.4	-
37.24	Cadina-1(2),4-diene	0.6	-
37.32	Curcumene	0.3	-
37.44	Germacrene D	0.1	-
37.56	α-Selinene	-	2.8
37.59	α-Bergamotene	1.4	-
37.83	α -Farnesene	3.2	-
38.09	Sesquiphellandrene	0.5	-
38.23	Trans-γ-Bisabolene	0.6	-
38.74	Elemol	-	0.2
39.06	Germacrene B	0.1	-
39.63	α-Patchoulene	0.4	-
40.00	β-Maaliene	-	1.4
40.19	α-Longipinene	0.2	-
40.68	β-Bisabolene	0.2	-
38.74	Elemol	-	0.2
38.87	Nerolidol 2	14.0	-
40.30	β-Selinenol	-	5.0
40.53	α-Bisabolol	3.2	-
40.96	Farnesol	0.4	-
	Monoterpene Hydrocarbones	33.4	14.7
	Oxygenated Monoterpenes	4.0	71.4
	Sesquiterpene Hydrocarbones	13.3	4.2
	Oxygenated Sesquiterpenes	17.6	5.2
	Total	**68.3**	**95.5**

Antimicrobial activities of *A. kotschyi* subsp. *kotschyi*

The antimicrobial activity of essential oil was evaluated by the disc diffusion method revealed variable levels of susceptibility in the tested bacteria and fungi (Table 2). The Gram negative *S. enteritidis* was most sensitive (40.5 mm) and displayed higher susceptibility than that to the antibiotic Norfloxacine (10 µg) (31.7 mm). Moderate

susceptibility was noted with each of Gram positive *E. faecalis* (10.3 mm) and *S. aureus* (11.2 mm), while the oil with the tested concentrations failed to show any effect on the Gram negative *E. coli*. Among the three tested fungi, only *A. fumigates* (16.2 mm) was susceptible to the oil at a compatible extent to that of the fungal antibiotic Nystatin (100 µg) (12.2 mm). MIC values determined by mean of agar dilution method were 5 µl in *S. enteritidis*, 10 µl in *S. aureus* and *A. fumigatus*, and 25 µl *in E. faecalis*.

Table 2: The mean ±SD growth inhibition zone and MIC/MFC values of the essential oil of *A. kotschyi* subsp. *kotschyi* growing wild in Lebanon.

Microorganisms Types		***A. kotschyi* subsp. *kotschyi***	**Norfloxacine 10 µg**	**Nystatin 100 µg**	***A. kotschyi* subsp. *kotschyi***
		Growth Inhibition Zone(mm)			**MIC (µl)**
Gram Positive Bacteria	*E. faecalis*	10.3±0.62	25.2±14.2	-	25.0
	S. aureus	11.2±0.94	9.2±5.5	-	10.0
Gram NegativeBacteria	*E. coli*	R	29.3±16.4	-	-
	S. enteritidis	40.5±23.4	31.7±19.4	-	5.0
Fungi	*A. fumigatus*	16.16±8.9	-	12.16±6.7	10.0
	T. mentagrophytes	R	-	R	-
Yeast	*C. albicans*	R	-	9.0	-

Results obtained from disc diffusion method, followed by the measurements of MIC indicate that the gram negative bacteria *S. enteritidis* and the fungus *A. fumigatus* were most sensitive to the essential oil among the tested microorganisms, both of which exhibited higher susceptibility to the oil than to the tested antibiotics Norfloxacine and Nystatin, respectively**.** However, the tested oils failed to show any activity against the Gram negative *E. coli* or in either of *T. mentagrophytes* and *C. albicans*. These noted variations in the obtained antimicrobial response may be explained by the structural differences of the cell envelopes, cellular enzymatic activities, and mode of action of essential oil [27-33]. Due to their hydrophobicity, *Achillea* essential oils and their components are most likely to exert their antimicrobial ability by the partition of the lipids of the bacterial and fungal cells membrane and mitochondria, disturbing the cells structures and rendering them more permeable [34-35]. Furthermore, this permeability is reported to vary between Gram positive and Gram negative bacteria depending on the reaction of essential oil with protein layer found as mucopolysaccharides and peptidoglycans in bacterial cell envelopes. The cell membrane of Gram positive bacteria contains more mucopolysaccharides, proteins and less phospholipids, whereas, Gram negative have more phospholipids. Thus, the permeability and effect of antimicrobial agents is expected to be more efficient on Gram positive bacteria [36]. However, our results are partially in contrast with previously reports indicating that Gram positive are more susceptible to essential oils that Gram negative bacteria [37-38]. These results of the antimicrobial assays indicated that the essential oil of our plant exhibited a higher activity against Gram negative tested bacterial strains of *S. enteritidis* while the Gram positive strains of *S. aureus* and *E. faecalis* displayed moderate activities.

Due to their potential pharmaceutical preparations, cosmetics and foods, numerous investigations on the effect of *Achillea* species have indicated a wide range of antimicrobial activity which generally support the traditional use of the plant as effective treatment in many diseases. In this study, the fact that growth inhibition of *Achillea kotschyi subsp. kotschyi* oil was limited to only four out of the seven tested strains might indicate a narrow antimicrobial spectrum.

The higher percentage of oxygenated monoterpenes (71.4% of fruit oil) and monoterpene hydrocarbons (33.4% of flower oil) might explain the observed antimicrobial activity of the essential oil tested. This activity could also be due to the possible synergic interaction with oxygenated sesquiterpenes and sesquiterpene hydrocarbones (17.6% and 13.3%, respectively, of flower oil). Extensively studies on *Achillea* have demonstrated that its flavonoids [39-41, and sesquiterpene lactones [41-43] possess antimicrobial properties. In particular, 1,8-cineole, camphor and borneol have been found to have a pronounced antimicrobial activity [44-45] and a more specific association with 1,8-cineole was indicated in *A. kotschyi* [20]. However, the biocidal activity of 1,8-cineole, *p*-cymene and camphor pure compounds have been reported as significantly weaker than the unfractionated essential oil of *A. biebersteinii* suggesting that minor compounds may probably be the active principles responsible for certain bioactivities [11]. It is also possible that minor compounds may act together in synergy to contribute to the bioactivity of the totality of essential oils [46]. In addition, β-thujone as the dominant constituent of the fruit oil herein tested, *A. multifida* and many essential oils rich in thujone (*Salvia* and *Thuja* species) possessing strong antimicrobial properties could

contribute to the inhibitory effect against tested microorganisms [47-48]. Nevertheless, antagonism among constituents should not be excluded when evaluating the oils bioactivities [24].

Conclusion

From the above discussion, it can be concluded that essential oil of *A. kotschyi* subsp. *kotschyi* of Lebanon varied in its constituents; β-thujone, nerolidol 2, β-terpinene, β-myrcene, 1,8-cineole, camphene, followed by trans-β-farnesene, α-bisabolol, sabinene and α-pinene. Oxygenated monoterpenes and monoterpenes hydrocarbons were predominant to oxygenated sesquiterpenes and sesquiterpenes hydrocarbons. The observed *in-vitro* activity might also be due to synergistic interaction between all components of the essential oil. Considering the increase development of resistance of bacteria, fungi and yeast to antibiotics, the present investigation together with previous studies provide support to the antibacterial properties of this plant oil. This study is the first report on essential oil composition and antimicrobial activity of the Lebanese A. *kostchyi* subsp. *kostchyi* and calls for further investigations to elucidate the effects of the oil and extracts on other biological activities. These findings confirmed that the essential of the plant can be different in quantity and quality according to geographical and environmental conditions and the period of plant growth. Thus, to obtain uniform contents we recommend that the plants from Lebanon should be grown in cultural conditions as the next step. The present investigation of the essential oil of A. *kostchyi* subsp. *kostchyi* support the traditional use of this plant in Lebanese folk medicine as antimicrobial for wounds treatment and its potential as a good source for new therapeutic agents.

References

1. Benedek, B., Kopp, B., and Melzig, M. F. (2007). *Achillea millefolium* L. sl–Is the anti-inflammatory activity mediated by protease inhibition. *Journal of Ethnopharmacology*, *113*(2): 312-317.
2. Euro+MedPlantBase - the information resource for Euro-Mediterranean plant diversity. Published on the Internet http://ww2.bgbm.org/EuroPlusMed/ (September 2015).
3. Mouterde P. (1966, 1970, 1983). Nouvelle Flore du Liban et de la Syrie. El Machreq. Editeurs :Beyrouth, distribution Librairie orientale.
4. Agar, T. and Engür, S. (2014). Evaluation of the wound healing potentials of three *Achillea* species on cultured NIH3T3 fibroblasts. *Second International Conference and Exhibition on Pharmacognosy, Phytochemistry and Natural Products*. China.
5. Albayrak, S. (2013). The Volatile Compounds and Bioactivity of *Achillea sieheana* Stapf. (Asteraceae). *Iranian Journal of Pharmaceutical Research, 2* (1): 37-45.
6. Hammad, H. M., Litescu, S. C., Matar, S. A., Al-Jaber, H. I., and Afifi, F. U. (2014). Biological activities of the hydro-alcoholic and aqueous extracts of *Achillea falcata* L. (Asteraceae) grown in Jordan. *European Journal of Medicinal Plants, 4*(3): 259-270.
7. Serdar, G., Sökmen, M., Demir, E., Sökmen, A., and Bektaş, E. (2015). Extraction of antioxidative principles of *Achillea biserrata* M. Bieb. and chromatographic analyses. *International Journal of Secondary Metabolite*, *2*(2): 3-15.
8. Tohme, R., Al Aaraj, L., Ghaddar, T., Gali-Muhtasib, H., Saliba, N. A., and Darwiche, N. (2013). Differential growth inhibitory effects of highly oxygenated guaianolides isolated from the Middle Eastern indigenous plant *Achillea falcata* in HCT-116 colorectal cancer cells. *Molecules, 18*(7): 8275-8288.
9. Al Sohaili, S. A., and Al-fawwaz, A. T. (2014). Composition and antimicrobial activity of *Achillea fragrantissima* essential oils using food model media. *European Scientific Journal, 10* (30): 1857 – 7881.
10. Çakır, A., Özer, H., Aydın, T., Kordali, Ş., Çavuşoglu, A. T., Akçin, T., and Akçin, A. (2015). Phytotoxic and Insecticidal Properties of Essential Oils and Extracts of Four *Achillea* Species. *Records of Natural Products, 10* (2): 154-167.
11. Tabanca, N., Kirimer, N., Demirci, B., Demirci, F., and Baser, K. H. C. (2001). Composition and antimicrobial activity of the essential oils of *Micromeria cristata* subsp. *phrygia* and the enantiomeric distribution of borneol. *Journal of Agricultural and Food Chemistry, 49*(9): 4300-4303.
12. Turkmenoglu, F. P., Agar, O. T., Akaydin, G., Hayran, M., andDemirci, B. (2015). Characterization of Volatile Compounds of Eleven *Achillea* Species from Turkey and Biological Activities of Essential Oil and Methanol Extract of *A. hamzaoglui Arabacı* and *Budak*. *Molecules, 20*(6): 11432-11458.
13. Dastjerdi, L. S., and Mazoji, A. (2015). Comparative chemical composition of the essential oils of Iranian *Achillea oxyodonta* from different ecological regions. *Journal of Applied Pharmaceutical Science, 5*(05): 106-109.

14. Magiatis, P., Skaltsounis, A. L., Chinou, I., and Haroutounian, S. A. (2002). Chemical composition and in-vitro antimicrobial activity of the essential oils of three Greek *Achillea* species. *Zeitschriftfür Naturforschung C*, *57*(3-4): 287-290.
15. Bozin, B., Mimica-Dukic, N., Bogavac, M., Suvajdzic, L., Simin, N., Samojlik, I., and Couladis, M. (2008). Chemical composition, antioxidant and antibacterial properties of *Achillea collina* Becker ex Heimerl s.l. and *A. pannonica* Scheele essential oils. *Molecules*, *13*(9): 2058-2068.
16. Bruno, M., Rosselli, S., Raccuglia, R. A., Maggio, A., Senatore, F., Arnold, N. A., and Herz, W. (2003). Terpenoids and flavones from *Achillea falcata* (Asteraceae). *Journal of the Mexican Chemical Society*, *47*(2): 130-131.
17. Senatore, F., Napolitano, F., Apostolides Arnold, N., Bruno, M., and Herz, W. (2005). Composition and antimicrobial activity of the essential oil of *Achillea falcata* L. (Asteraceae). *Flavour and fragrance journal*, *20*(3): 291-294.
18. Bader, A., Flamini, G., Cioni, P. L., and Morelli, I. (2003). Essential oil composition of *Achillea santolina* L. and *Achillea biebersteinii* Afan. collected in Jordan. *Flavour and Fragrance Journal*, *18*(1): 36-38.
19. Walter, K. S., and Gillett, H. J. (1998). *1997 IUCN Red List of Threatened Plants*. IUCN.
20. Karamenderes, C., Karabay, N. Ü., and Zeybek, U. (2002). Composition and Antimicrobial Activity of the Essential Oils of Some *Achillea* L. Species in Turkey Turkiye'dekibazi Achillea L. Taksonlarimn Uçucu Yaglarmm Binimive Antimikrobiyal Aktivitesi. *Acta Pharmaceutica Turcica*, *44*: 221-225.
21. Karaalp, C., Yurtman, A. N., and KarabayYavasoglu, N. U. (2009). Evaluation of antimicrobial properties of *Achillea* L. flower head extracts. *Pharmaceutical Biology*, *47*(1): 86-91.
22. NCCLS. (1997). Performance Standards for Antimicrobial Disk Susceptibility Tests: Approved Standard M2-A7. National Committee for Clinical Laboratory Standards, Wayne, PA, USA.
23. Bader, A., Panizzi, L., Cioni, P. L., and Flamini, G. (2007). *Achillea ligustica*: composition and antimicrobial activity of essential oils from the leaves, flowers and some pure constituents. *Central European Journal of Biology*, *2*(2): 206-212.
24. Nemeth, E., and Bernath, J. (2008). Biological activities of yarrow species (*Achillea* spp.). *Current Pharmaceutical Design*, *14*(29), 3151-3167.
25. Naser, S. M., Thompson, F. L., Hoste, B., Gevers, D., Dawyndt, P., Vancanneyt, M. and Swings, J. (2005). Application of multilocus sequence analysis (MLSA) for rapid identification of Enterococcus species based on rpoA and pheS genes. *Microbiology, 151:* 2141–2150.
26. Hold, K. M., Sirisoma, N. S., Ikeda, T., Narahashi, T., and Casida, J. E. (2000). α-Thujone (the active component of absinthe): γ-Aminobutyric acid type A receptor modulation and metabolic detoxification. *Proceedings of National of Sciences of the United States of America*, *97*(8): 3826-3831
27. Hammer, J., Valsasnini, P., Tolba, K., Bolin, D., Higelin, J., Takacs, B., and Sinigaglia, F. (1993). Promiscuous and allele-specific anchors in HLA-DR-binding peptides. *Cell*, *74*(1): 197-203.
28. Dorman, H. J. D., and Deans, S. G. (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology*, *88*(2), 308-316.
29. Bagamboula, C. F., Uyttendaele, M., and Debevere, J. (2004). Inhibitory effect of thyme and basil essential oils, carvacrol, thymol, estragol, linalool and p-cymene towards *Shigella sonnei* and *S. flexneri*. *Food Microbiology*, *21*(1): 33-42.
30. Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology*, *94*(3): 223-253.
31. Gordon Jr, D. C., and Prouse, N. J. (1973). The effects of three oils on marine phytoplankton photosynthesis. *Marine Biology*, *22*(4): 329-333.
32. Mahmoud, S. S., Williams, M., and Croteau, R. (2004). Cosuppression of limonene-3-hydroxylase in peppermint promotes accumulation of limonene in the essential oil. *Phytochemistry*, *65*(5): 547-554.
33. Guesmi, A., and Boudabous, A. (2007). Activité antimicrobienne de cinq huiles essentielles associées dans les produits de thalassothérapie. *Revue des Régions Arides*, 1: 224-230.
34. Degryse, A. C., Delpla, I., and Voinier, M. A. (2008). Risques et bénéfices possibles des huiles essentielles. *Rapport de stage en vue de l'obtention du diplôme d'ingénieur du génie sanitaire*.
35. Nogueira, J. H., Gonçalez, E., Galleti, S. R., Facanali, R., Marques, M. O., and Felício, J. D. (2010). Ageratum conyzoides essential oil as aflatoxin suppressor of *Aspergillus flavus*. *International Journal of Food Microbiology*, *137*(1): 55-60.
36. Al-Saimary, I. E., Bakr, S. S., Khudaier, B. Y., and Abass, Y. (2007). Efficiency of antibacterial agents extracted from *Thymus vulgaris* I. (Lamiaceae). *The Internet Journal of Nutrition and Wellness*, *4* (1).

37. Ouattara, B., Simard, R. E., Holley, R. A., Piette G. J., and Begin, A. (1997). Antibacterial activity of selected fatty acids and essential oils against six meat spoilage organisms. *International Journal of Food Microbiology*, 37: 155-162.
38. Mangena, T., and Muyima, N. Y. O. (1999). Comparative evaluation of the antimicrobial activities of essential oils of *Artemisia afra, Pteronia incana and Rosmarinus officinalis* on selected bacteria and yeast strains. *Letters in Applied Microbiology*, *28:* 291-296.
39. Marchart, E., and Kopp, B. (2003). Capillary electrophoretic separation and quantification of flavone-O- and C-glycosides in *Achillea setacea* W. et K. *Journal of Chromatography B, 792*(2): 363-368.
40. Cushnie, T.P.T., and Lamb, A. J. (2006). "Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents*, *2*(27): 181.
41. Benedek, B., Rothwangl-Wiltschnigg, K., Rozema, E., Gjoncaj, N., Reznicek, G., Jurenitsch, J., and Glasl, S. (2008). Yarrow (*Achillea millefolium* L. sl): pharmaceutical quality of commercial samples. *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, *63*(1): 23-26.
42. Todorova, M., andTsankova, E. (2001). Sesquiterpene lactones from *Achillea chrysocoma* and *Achillea coarctata*. *Zeitschrift für Naturforschung C, 56*(11-12): 957-960.
43. Werner, I., Mucaji, P., Presser, A., and Glasl, S. (2007). Sesquiterpenes and Phenolic Compounds from *Achillea clypeolata* Sesquiterpenes and Phenolic Compounds from *Achillea clypeolata*. *Zeitschrift für Naturforschung B*, *62*(2): 267-271.
44. Nemeth, E. (2005). Essential oil composition of species in the genus *Achillea* .*Journal of Essential Oil Research*, *17*(5): 501-512.
45. Radulović, N., Mišić, M., Aleksić, J., Đoković, D., Palić, R., and Stojanović, G. (2007). Antimicrobial synergism and antagonism of salicylaldehyde in *Filipendula vulgaris* essential oil. *Fitoterapia*, *78*(7): 565-570.
46. Lahlou, M. (2004). Methods to study the phytochemistry and bioactivity of essential oils. *Phytotherapy Research*, *18*(6): 435-448.
47. Boser, K. H. C., Demirci, B., Demirci, F., Kocak, S., Akinci, C., Malyer, H., and Guleryuz, G. (2002). Composition and antimicrobial activity of the essential oil of *Achillea multifida*. *Planta Med. 68*(10): 941-943.
48. Tsiri, D., Graikou, K., Pobłocka-Olech, L., Krauze-Baranowska, M., Spyropoulos, C., and Chinou, I. (2009). Chemosystematic value of the essential oil composition of Thuja species cultivated in Poland—antimicrobial activity. *Molecules*, *14*(11): 4707-4715.